EXPERIMENTAL ARTHROPOD TRANSMISSIONS OF TULAREMIA

by

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Although research into the problem of arthropod transmission of tularemia has undergone great development since McCoy (1911) first reported infected fleas, no quantitative methods for transmission studies have previously been devised. Throughout the course of earlier research, quantitative methods only have been applied. It is not desired to discredit the work of other workers from this point of view since it is believed that the information gained from their experiments is of profound importance and forms the basis for future investigations. On the other hand, if further information is to be obtained regarding arthropod transmission of this disease quantitative studies must be undertaken. For instance, the number of organisms retained by a vector after an infective feeding and the variation of this number within a species has never been determined. The question as to whether the infectious organisms within an arthropod diminishes after prolonged periods of starvation has often caused much speculation. Little is known concerning the fate of Bacterium tularense within an arthropod vector which feeds upon naturally resistant, recovered, or vaccinated hosts. What information has been accumulated on this subject resulted from the investigations of Bell (1945) and since this author's work was accomplished on vaccinated rabbits, it seems probable that a study with the above mentioned types of hosts would reveal information of much significance, particularly if different vectors were used.

With these objectives in mind the present study was undertaken and quantitative methods were employed whenever feasible. Inasmuch as this study was carried out with two different arthropod vectors and since different experiments were undertaken the results of this study are divided into two parts.

MITE TRANSMISSION

With the exception of the work reported by Francis and Lake (1922) no information is available in this country concerning the ability of mites to act as vectors of Bacterium tularense. These authors conducted an incidental experiment in which 10 mites of the species Liponyssus isabellinus were recovered from a white mouse dead from a laboratory infection of tularemia. "The mites were rubbed in a mortar with saline solution and the suspension was injected subcutaneously into a white mouse, causing its death in 4 1/2 days, with typical lesions of tularemia."

In Russia, more numerous observations are available. Volferz, et al (1934) conducted investigations in the Stalingrad Steppes and in one case recovered a spontaneous infection of tularemia in gamasid mites. These mites were collected in an abandoned nest of a hibernating mouse-like rodent. Grzhebina (1939) studied the relationship of gamasid mites and human cases of tularemia which occurred among personnel occupied in the trapping of water rats. On

epidemiological evidence this author supposed that these mites might serve as the origin of infection for these people. Grzhebina also reported that Laelaps echidninus, Laelaps pachypus, and Eulaelaps stabularis removed at death from infected water rats were capable of retaining Bacterium tularense for 15 days at 6 to 10° C. and for a period of 10 days when kept at room temperature (18 to 24° C.). This author found that on transferring mites presumed to be infected to non-infected water rats, white mice and guinea pigs, only one positive transmission was observed. Olsoufiev (1943) recovered infected mites, mainly Haemolaelaps sp., from nests of Microtus arvalis. He also noted a "number of fresh corpses of these rodents dead of tularemia." This author reported that gamasid mites were found capable of retaining the infection for an extremely short period of time, 31 hours at a temperature of from 23 to 26° C.

Inasmuch as parasitic mites are abundant on vertebrates, especially the rodents which play an important role in the maintenance of tularemia in nature, and since the above review indicates that little is actually known concerning the relationship of mites and disease, it was believed that an experimental study would help lay a foundation for an understanding of this condition. The tropical rat mite Liponyssus bacoti (Hirst 1913) was used as the vector in this phase of the study.

MATERIALS AND METHODS

Two stock colonies of the tropical rat mite were established, one from Swiss white mice infested on arrival at the laboratory and the second from specimens furnished by J. Allen Scott, University of Texas Medical School, Galveston. Progeny from these two colonies provided mites for all subsequent experiments.

These colonies were maintained in aluminum cages 10 x 10 x 9 inches with mesh wire tops. Fine wood shavings approximately three inches deep provided litter for mites and mice. The mouse cages were placed in white enamel pans which were filled to a depth of two inches with water. The enamel pans (Plate I) were in turn placed in large metal receptacles containing a 5 per cent solution of phenol to kill the mites which occasionally escaped from the water moat of the enamel pan. The water moat was used to obtain mites for experimental purposes as many engorged protonymphs and adults tended to migrate from the litter of the cage.

In subsequent experiments dealing with infected colonies the same procedure was carried out, except that the moat in the enamel pan consisted of water for a period of 12 hours only when mites were desired for samplings. During the remainder of the time this moat contained the phenol solution like the outer pan.

Swiss white mice obtained from the Maple Grove Rabbitry were used as the experimental animal. If mites were to be infected,

Plate I. Method used in maintaining mite colonies.

5.

these animals were injected with 0.5 ml of a 10^{-7} saline suspension of the virulent Sm strain of Bacterium tularense. The 10^{-7} suspension was checked in a photolometer and as a further check plate cultures were made at this dilution to ensure that 200 to 400 organisms were present per ml. Since the mites were not allowed to feed upon the mice until 72 hours had elapsed from the time of inoculation, these plate cultures were checked and any mice infected with a suspension not giving satisfactory plate counts were discarded. It should be mentioned, however, that only rarely were the mice discarded for this reason. The mites were fed 72 hours after inoculation because the mice died within 1 to 12 hours after this time and previous experience indicated that a consistently high percentage of the mites could be infected at this time.

Plate II illustrates the method by which the mites were fed. The mouse was confined in a small wire mesh holder, its tail being threaded through a hole in a rubber stopper and sealed at the base with modeling clay around the tail and stopper. If the mites were to feed on infected mice the tail was washed with alcohol, the end clipped and a drop or so of blood was placed on culture medium to make certain Bacterium tularense was circulating in the peripheral blood at the time of feeding. The mites were kept in glass cylinders 9 x 30 mm, both ends of which were tightly stoppered with cotton plugs. After jarring the mites to one end and removing the

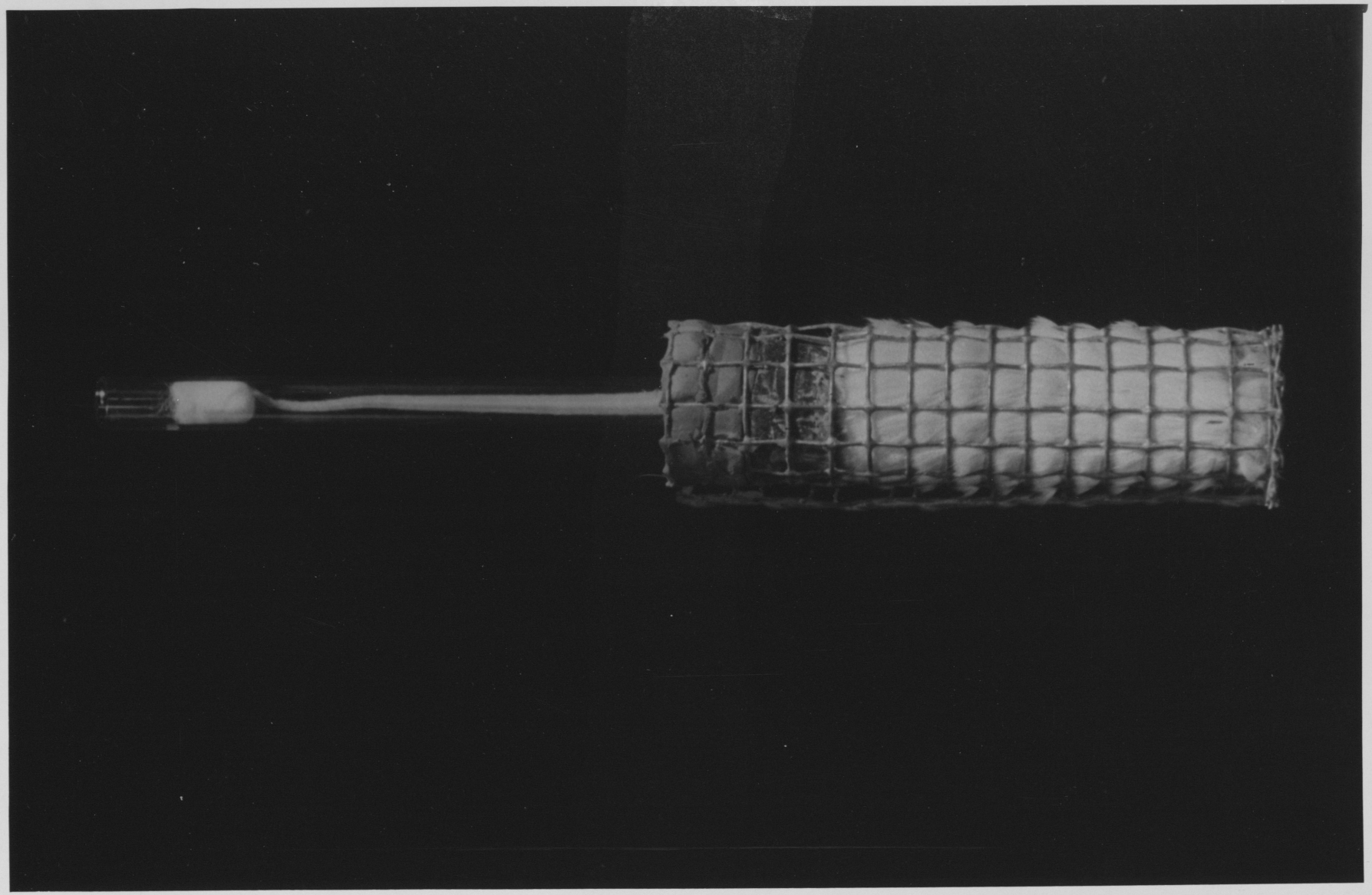

Plate II. Method of feeding mites (dorsal view).

plug from the opposite end of the tube, the mouse's tail was inserted. The glass cylinder was then pushed firmly into the base of modeling clay. Upon completion of feeding the mites usually collected at the end of the tube with the cotton plug. By gently removing the tube from the clay the mouse's tail was withdrawn and the tube stoppered without excape of the mites. Unless stated otherwise, the engorged mites were placed in humidifying jars containing a saturated solution of ammonium chloride which gave a relative humidity of 85 per cent.

The observations made concerning the life cycle agreed with those of Bertram et al (1945) and Skaliy and Hays (1949). By way of review it should be mentioned that once fully engorged, the protonymphs moult within 24 to 48 hours to the non-feeding deutonymph stage, and thence to the adult stage in the same period of time. It was noted that one to several feedings were required for the protonymphs before they moulted to the succeeding stages. However, after the second opportunity to feed, approximately 40 per cent of the protonymphs would engorge completely, at which time they were separated from the remaining partially engorged protonymphs and the adults. The separation was accomplished by slightly anesthetizing the mites with chloroform, removing one of the cotton plugs and placing the mites in a watch glass partially filled with water. With gentle tapping the majority of the mites were forced

from the tube into the water. The engorged protonymphs were then removed by means of a small spatula made from stainless steel wire and stored in clean tubes, the number per tube depending upon the experiment for which the mites were to be utilized.

During the course of the study it seemed that much less difficulty was incurred in feeding either the protonymph or the adults by the method described as compared to the methods used by Bertram et al (1945) and Skaliy and Hays (1949). Since the tail of the mouse has a much more delicate cover than that of the rat only slight scraping of the skin was necessary to remove the superficial scales to induce the protonymphs to feed. In fact, many times protonymphs were observed feeding on normal areas of the tail and they engorged at approximately the same rate of speed as those feeding on the scrapped area. Usually no difficulty was encountered with excess fluid oozing from the scraped area of the mouse's tail if done carefully and since protonymphs were found to engorge on the unscarified tail the above practice was discontinued in later experiments.

In making the quantitative plate counts the mites were first immersed in a 5 per cent solution of phenol for 15 minutes and then rinsed 3 times in normal saline. This procedure was carried out in an attempt to cut down contamination as much as possible in the plate cultures made at the lower dilutions. After the last washing

in saline the mites were stored in sterile tubes and within a few hours the following procedures were carried out. Each mite was ground in 1 ml of sterile normal saline in a sterile mortar and pestle. This suspension was considered as 10^{-0} from which 0.1 ml was transferred to make serial 10 fold dilutions up to 10^{-4}. Plate cultures were made at 10^{-0}, 10^{-2}, and 10^{-4}. The glucose cysteine blood agar was used and the method of quantitative plate counts was essentially the same as that reported by Downs et al (1947). Five tenths of a ml of the original 10^{-0} suspension was injected intraperitoneally into a white mouse as a more delicate test than culture for the presence of organisms. The mice were checked twice daily for a period of 15 days. In case the plates from the mites were negative and the injected mice died, these mice were autopsied and plate cultures were made of the spleen and heart blood. Those mites containing only a sufficient number of organisms to produce death in the mice were regarded as having a very low number of organisms and in compiling the data for the number of organisms per mite they were listed as positive with no number of organisms indicated since it was believed that number was too low to be of importance. All plate cultures were checked at 48 hours and the plates appearing negative or doubtful were held 24 hours longer before discarding. In instances where plate counts were doubtful, a gram stain was made and as a further check mice were injected with a saline

suspension of the organisms. Fortunately this condition was rarely encountered.

RESULTS

Inasmuch as no information was available concerning the abilities of the tropical rat mite as a vector of tularemia, preliminary experiments were conducted to establish this fact: Adult mites were allowed to feed on an infected mouse and one week after engorgement three pools of 10 mites each were ground in 2 ml of normal saline and 0.5 ml injected into each of two white mice for each respective pool. All mice were dead within 48 hours and the plate cultures made from the spleen and heart blood were identified as positive for Bacterium tularense. A similar experience occurred when mites were infected as protonymphs by feeding on infected mice and allowed to moult to the adult stage before being ground and injected in the above manner. This indicated the mites infected as protonymphs retained the infection through the successive moults to the adult stage.

Having established that the mites could be infected with Bacterium tularense it seemed advisable to establish the number of organisms per mite and the percent which became infected. To obtain this information 100 mites infected as protonymphs by feeding on the tail of infected mice were used. Four days after reaching the adult

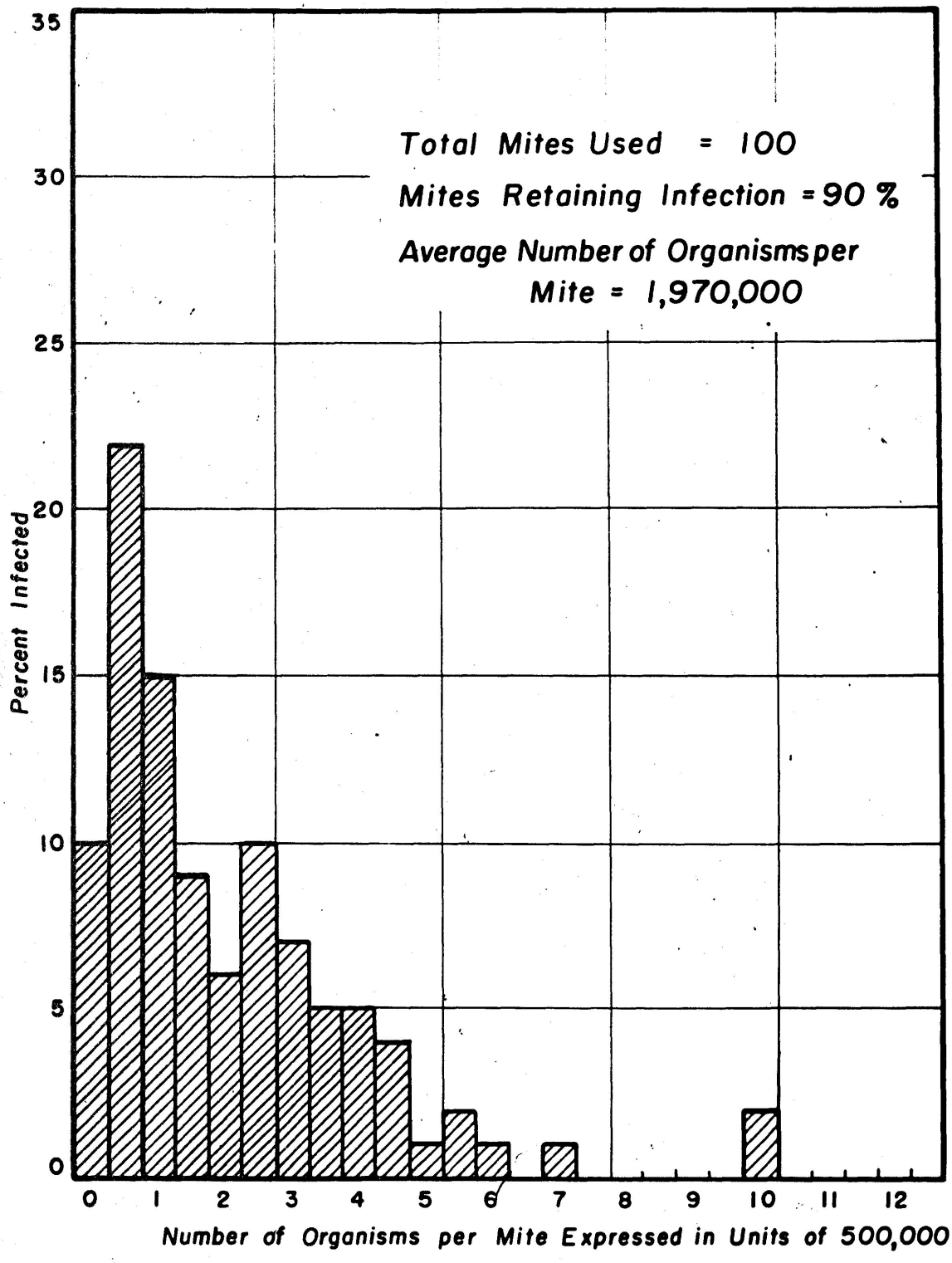

Fig. 1. Variation of the number of organisms in mites after one infective feeding.

stage the mites were ground individually, plate cultures made at the specified dilutions and mice injected as a more delicate test than culture.

Ninety per cent of the mites proved to be infected and figure 1 shows the variation in the number of organisms per mite. The minimum and maximum number of organisms per mite was 100 and 10,000,000 respectively. The average number of organisms for the infected mites was 1,975,000. A small number of plates were contaminated at the 10^{-0} dilution but this contamination was lost at the higher dilutions. Two percent of the total mites used were positive only by mouse inoculation.

To determine the length of survival of Bacterium tularense in unfed mites, protonymphs were infected and after moulting to the adult stage 200 were removed and placed in storage tubes. These tubes were stored at room temperature and placed in humidifying jars with a relative humidity of 85 per cent. Twenty days after engorgement, or approximately 20 days after reaching the adult stage ten mites were still alive. These mites were ground, with plate cultures and mouse inoculations being made in the usual manner.

Table I shows the variation in the number of organisms per mite and the number of days required to produce death in the mice. Two mice apparently were negative since the plate cultures were

TABLE I

Number of organisms in individual mites.

Mite No.	No. Organisms Per Mite	Mouse No.	No. of Days Before Death	Autopsy Plates
1	3,400,000	1	2	
2	1,000,000	2	3	
3	5,200,000	3	2	
4		4		N*
5		5	7	P*
6	8,000,000	6	2	
7		7		N
8	2,500,000	8	2	
9		9	6	P
10	12,000	10	4	

* N indicates negative cultures at autopsy. *P indicates positive cultures.

negative and the mice were discarded as normal after observation for 15 days. Mites 5 and 9 were positive only by mouse inoculation as verified by cultures made of heart blood and spleen. Therefore, 80 per cent of the mites proved to be infected with an average of 2,500,000 organisms.

To determine the possibility of transmission of Bacterium

tularense by biting, mites were infected as protonymphs and allowed to fast 4 to 5 days in the adult stage before being fed upon normal mice. The mites were placed in tubes with 20 mites per tube and were fed at intervals of 5 days on the tails of non-infected mice. On completion of feeding, the mice were given the same number as the mite tube and then placed in cages for the usual period of observation. In the event death occurred the mice were autopsied and the usual plate cultures were made. Six days after the completion of the last feeding the mites were ground individually in saline and mouse inoculations made to determine the number of mites infected. In the event that death occurred beyond the fifth day in these mice, plates were made of the spleen and heart blood for positive identification of Bacterium tularense.

Two mice died out of the total of 30 used and the cultures in both cases were negative. As is shown in Table II, 169 or 84.5 per cent of the 200 mites used were found to be infected. Since a relatively high percent of the mites were found to be infected and no positive evidence of transmission took place it is assumed that if the mite does transmit tularemia by this method it is very rare and that another more logical method of infecting the host must be found.

TABLE II

Attempted transmission of tularemia to mice by the bite of mites.

No. of Mite Tube & Mouse	No. of Mites Per Tube	Total Mice Dead After 3rd Feeding	Autopsy Plates Neg. or Pos.	No. of Mites Infected Per Pool
1	20			16
2	20			19
3	20	1	N*	17
4	20			15
5	20			18
6	20			17
7	20	1	N	19
8	20			16
9	20			18
10	20			14
Total	200	2	2	169

* N indicates negative cultures at autopsy.

In infected colonies handled in the same manner as the normal stock colonies, mice usually died within a period of 5 days after being placed with the mites. Cultures made of spleen and heart blood in all instances were positive for tularemia. Observations

indicated that the mice attempted to free their coats by biting etc. At times the area around the mouth of the mice appeared to be red with blood from crushing engorged mites. To determine if mites could be infected orally with crushed mites, 20 groups of 10 female mites each were fed on the tails of infected mice 72 hours after innoculation. After feeding, the mites in each group were ground in 2 ml saline and 0.5 ml given orally by a dropper to each of two mice which had been forced to thirst for a period of 48 hours. One-tenth ml of the saline suspension was spread on a culture plate as a control to ensure that the mites used were infected. The mice were then allowed a normal diet and within 4 days all mice were dead. All plate cultures made on death of the mice, and those made at the time of feeding from the saline suspensions were positive for Bacterium tularense. These results indicate that the mites do not transmit the infection by biting, but that the mice can become infected by crushing the mites orally. Francis and Lake (1922) established that a susceptible animal could infect itself by the ingestion of infected bedbugs.

Transovarian transmission of two diseases has been reported of the tropical rat mite. Dove and Shelmire (1931) reported trans-ovarian transmission of typhus. Philip and Hughes (1948) reported this type of transmission in experimental work with rickettsialpox. Since this method of transmission had been reported for these

diseases it seemed desirable to test the ability of the mites to transmit tularemia in this manner.

One hundred female mites which had received an infective feeding as protonymphs were separated from males after another infective feeding in the adult stage. These mites were placed individually in tubes, the tubes numbered and left at room temperature and humidity. Seventy-two hours later oviposition was apparently complete at which time the females were transferred to clean tubes, retaining the same number in the transfer. When the progeny for each respective female reached the protonymph stage, they in turn were placed in new tubes likewise retaining the original numbers. After the protonymphs had been stored in the new tubes for 48 hours the female mites were ground individually in 1 ml of normal saline. Five-tenths of a ml of this suspension was then injected intraperitoneally into a mouse which received the same number as the mite. The same technique was followed for the protonymphs except that the progeny of each female was ground and injected as a unit. All the mice were autopsied and the usual plate culture made of spleen and heart blood.

Seventy-nine of the adult mites proved to be infected. Of this group 17, or 21.5 per cent had progeny which also proved to be infected with Bacterium tularense. No attempts were made to demonstrate this type of transmission in the egg or larval stage since these immature forms of this particular mite do not feed.

Therefore, for all practical purposes transovarian transmission would not be of importance until the feeding protonymph stage of development was reached.

Assuming that transovarian transmission had been demonstrated above, further experimental work was undertaken to determine if protonymphs from infected adults could establish an infection in a colony of mice. For this experiment approximately 500 protonymphs, progeny of adults which proved to be 80 per cent infected, were placed in a cage with two mice and handled in the same manner as the normal stock colonies. The cage and litter were autoclaved before introducing the mites or mice. The protonymphs prior to being placed in the cage were cared for in the same manner as described above in the first phase of the transovarian study.

Ten days after the protonymphs had been placed in the cage, the first mouse died and on the eleventh day the second mouse was dead. Cultures on both mice proved to be positive and two more mice were then added to the colony. Within 5 days both mice were dead and again the cultures made at autopsy were positive which indicated the infection was well established. This same type of experiment was undertaken twice more with approximately the same number of protonymphs. The same results were experienced in the third colony, but in the second colony negative results were obtained which seemed to indicate this type of transmission could

be extremely variable.

To compare the effects of feeding on a normal host and a recovered host a series of female mites were given an infective feeding for the first time after reaching the adult stage. One series was then fed at intervals of 6 days on the tails of normal mice and the second group were fed at the same intervals on recovered mice. These recovered mice may be assumed to be solidly immune in view of the work of Downs and Woodard (1949). These authors have shown that mice which have recovered from an infection with a sublethal dose of a strain of Bacterium tularense of lowered virulence such as the "Jap" strain are able to survive as much as 10,000 LD_{50} of a fully virulent strain. Pannell (1950) has shown that the peak of circulating antibodies is reached within a period of 5 days in these normal mice. After this period of time the antibody level remained constant for 8 to 10 days and gradually declined. In further observations she found that if these mice were given a booster shot (0.5 ml at 10^{-2}) with the same strain an indentical peak of antibody production was produced. However this time the peak was reached in 24 to 48 hours and remained constant over a considerable length of time. Each mouse referred to as "recovered" had received an infective dose of the "Jap" strain, 0.5 ml of 10^{-4} dilution of a standard suspension. The mites were allowed to feed on these mice for the first time 9 days after receiving this

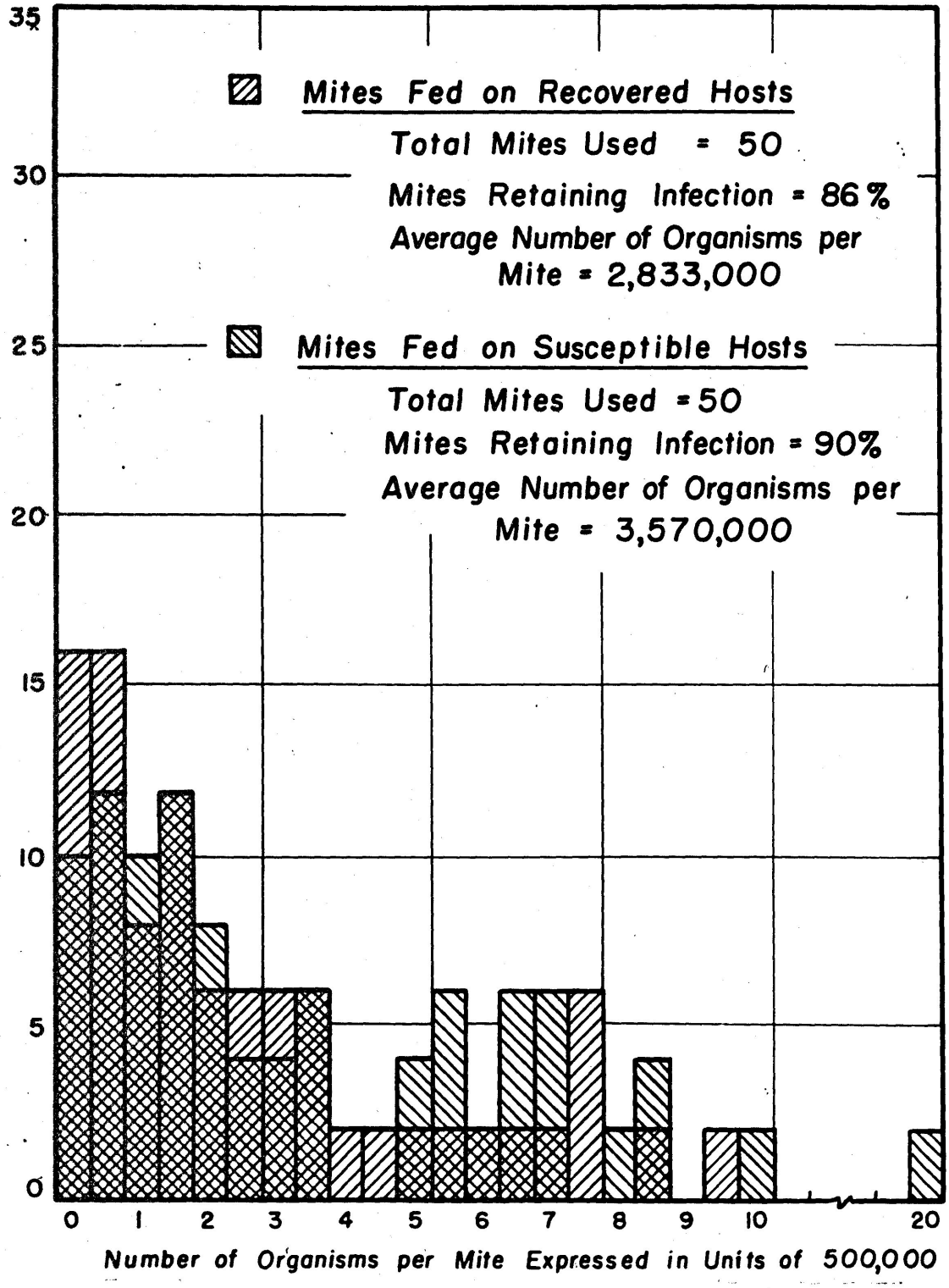

Fig. 2. Variation of the number of organisms per mite after two feedings on recovered and susceptible hosts.

quanity of organisms. Thirty-six hours before the second feeding took place the mice were given a booster shot. By feeding the mites at these intervals it was believed that the antibody level was near the top level and should, therefore, more clearly indicate the effects of feeding on this host. After each feeding the mice were identified according to the mites it had fed and observed for the usual period of 15 days with rountine techniques being carried out in the event of death. After the last feeding the mites fasted for 5 days at which time the mites were ground individually and quantitative plate counts were made.

Ninety and 84 per cent of the mites feeding on the normal and recovered mice respectively were found to be infected. Figure 2 gives the variation in the relative number of organisms per mite. The minimum and maximum number of organisms per mite for the series feeding on the normal host was 12,000 and 20,000,000 respectively while the average was 3,570,000. For those mites which fed on the recovered mice the minimum and maximum was 2,000 and 8,500,000 respectively and the average number of organisms was 2,833,000.

In order to observe the effects of mite passage on the virulence of the Sm strain, mouse LD_{50} titrations were made on organisms recovered from the plate cultures of three adult mites and from one pool of protonymphs. The group of protonymphs were unfed and were progeny from infected adults. One transfer was made from these

original plate cultures before the titration was accomplished. The determination of the LD_{50} was made according to the method of Muench (1938). Dilutions were made at 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10}. Six mice were used at each dilution and each mouse received 0.5 ml of the respective dilution. The results obtained were compared to known standard titrations in unpublished experiments of Downs (1950).

The titration of the organisms passaged through the three adult mites were $10^{-9.20}$, $10^{-9.36}$, and $10^{-9.75}$. The titration of the organisms believed to be transovarially transmitted was $10^{-8.62}$. The range in the LD_{50}'s from the three adult mites coincides with those titrations reported by the above author; that of the protonymph was slightly lower. It is believed that the slight decrease in titer for this particular titration is not of importance, however it may suggest that the organisms lose virulence in transovarian transmission.

To determine if the tropical rat mite could retain an infection under conditions somewhat similar to those in nature, two colonies of mites were infected by placing two mice in each colony. These mice had been injected with Bacterium tularense prior to this time. When these two mice died they were replaced in the cage by uninfected mice and at death these mice were replaced by new ones. However, since the mite population became excessive at times, mice were withheld from the cage for 10 to 14 days which usually

controlled the population readily. Periodically the mice were autopsied and the usual plate cultures were made to insure that the mice were dying with tularemia, and to parasitism of the mites alone.

After a period of nine months had elapsed since infecting the colony, the mice were withheld from the colonies for a period of 4 weeks. The mice which were placed in colony I after this interval of time did not die for a period of 12 days. These mice were autopsied and the cultures made from the spleen and heart blood were positive for Bacterium tularense. Those mice placed in colony II were still alive at the end of 5 weeks, at which time they were sacrificed and the usual plate cultures made which proved to be negative.

When the colonies had been maintained for a year's time, the cages contained a larger proportion of mouse feces than thought desirable. A series of mites from each colony was then transferred to clean cages and within a short time both colonies were again well supplied with mites. The mice from the first colony continued to die at the regular 5 day interval after being placed with the mites while the mice in the second colony died only after prolonged periods of time. Since the cultures made from the latter group of mice were always negative, it was assumed that gross parasitism by the mites themselves was probably responsible for their death and that the mites had lost their infection. For this reason 2 pools

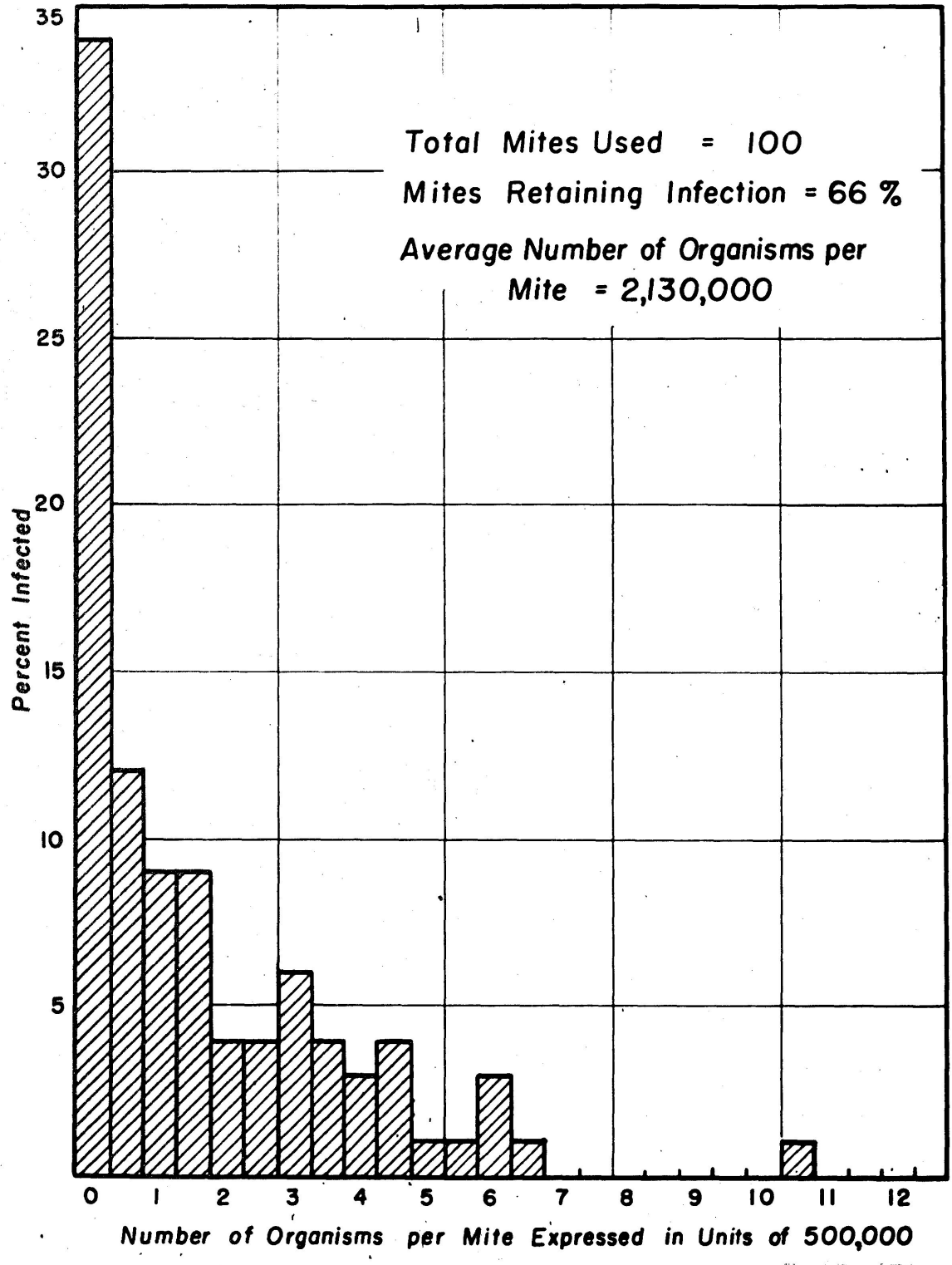

Fig. 3. Variation of the number of organisms in mites removed from a colony infected 18 months previously.

of 100 mites from colony II were ground in 3 ml of normal saline and 0.5 ml injected into each of 4 mice for each pool. The mice were observed for the usual 15 day period and since none of them died, they were discarded as normal. With the amount of negative findings compiled it seemed certain the mites from the second colony were no longer infected and this colony was then discontinued.

The first colony was retained until 18 months had elapsed from the time the mites were first infected. At this time 100 engorged mites were removed from the colony after fresh mice had been in the cage for 48 hours. This particular time was choosen as it was believed that this interval would give a clearer picture of the rate of infection and the number of organisms per mite than, for example, mites taken later when the mice were either ill or dying of tularemia. As the mites were removed they were stored in tubes for a period of 6 days at which time they were ground individually in saline with the routine procedures carried out for making the quantitative plate counts.

Sixty-five per cent of the mites used were infected, and figure 3 shows the variation in the relative number of organisms retained by the mites. The minimum and maximum number of organisms per mite was 1,000 and 10,500,000 respectively while the average number of organisms was 2,130,000. These figures are all in the

same range as those of previous experiments and suggests that the mites may be able to retain an infection so long as susceptible hosts are available.

DISCUSSION

The evidence obtained in the above experiments indicates that the tropical rat mite, infected in the protonymph stage of development transmits the infection to the deutonymph and thence to the adult; that once infected the mite remains so for the rest of its life despite several feedings on recovered or susceptible hosts. In certain instances the infection is passed on to the next generation, but the results in this case may be extremely variable. As to why the majority of the mites do not, and as to how the small percentage (21.5 per cent) transmit the infection transovarially is not known. How important transovarian transmission may be in the maintenance of this disease with reference to this mite has not been established. The last statement applies as well to many other instances where transovarian transmission has been reported for this disease.

Inasmuch as experimental work has shown that this mite is unable to transmit the infectious organisms by biting, a certain amount of its efficiency as a vector is lost. That the mice can become infected by crushing the mites orally has been clearly

demonstrated and while no actual experiments were undertaken to determine the following possibility, it seems probable that the mice in attempting to free themselves of the mites could crush an infected one over a broken area in the skin and thus produce an infection, particularly since this portal of entry is known for the rabbit to human cycle of this disease. It does not seem probable that this mite would produce infection by fecal contamination, at least to any great extent, since observations have shown this mite to be a clean feeder, and defecation usually takes place away from the host, particularly in the adult stage.

That the number of organisms within a series of mites may vary a good deal is evident. This variation appears to be as great in the mites fed under controlled conditions as in those which fed at random in the colonies, the only apparent difference being that the percentage infected seemed to be consistantly higher in the mites of the former group. The increase in the average number of organisms per mite after two feedings on either a susceptible host or two feedings on a recovered host as compared to one feeding on an infected host is more apparent than real. In fact, those mites which fed on the recovered mice were almost identical, in the number of organisms reported, to those mites which had one engorgement on an infected host. The difference in the average number of organisms and in the maximum range in numbers for mites of the susceptible

animal series was somewhat greater; however, this may not be significant in view of the wide range of variability. It was particularly interesting to note that the average number of organisms per mite in the colony which had been infected for 18 months was in close agreement to those mites fed under controlled conditions.

To judge from the mouse LD_{50} titrations, the effects of mite passage seems neither to increase nor to decrease the virulence of the Sm strain of Bacterium tularense. The one titration from infected protonymphs, although slightly lower than that from the adult mites, is not believed to be sufficient evidence to warrant a conclusion that transovarian passage may tend to lower the virulence of this particular strain.

With reference to the loss of infection in the second colony when attempting to test the ability of mite colonies to remain infected, no definite explanation can be given. However, it should be pointed out that the population of this colony was at a particularly low ebb after the prolonged fasting of 4 weeks, more so than that of the first colony which was able to retain its infection for 18 months. Although 65 per cent of this first colony was found infected, it is believed that this percentage could vary in either direction from 65. If it were considerably less than 65 per cent, and since the population was extremely low, it seems possible that the infected mites could have been present in such

low numbers that a colony of non-infected mites was established.

It may seem that this study using Liponyssus bacoti is a laboratory phenomenon without much practical application. This may be true; however, since transmission by this mite is possible, it seems likely that other closely related species can also be vectors. Since mites can retain the infection after prolonged periods of fasting and after feeding on recovered hosts, it may be presumed that mites can play a more important role as vectors than previously supposed. However, much data must be obtained, both experimentally and in nature, before this assumption is established.

TICK TRANSMISSION

Inasmuch as tick transmission of tularemia has been established by many investigators working with several different species and since an extensive review of this subject was presented by Steinhaus (1947) no detailed review of the literature seems necessary.

Amblyomma americanum (Linnaeus, 1758), commonly known as the "lone star tick," was used as the vector during the course of this study. Philip in unpublished experiments found that if this tick was infected during the larval stage, this infection was retained throughout the subsequent stages of that generation. He made no attempt to establish the possibility of transovarian transmission and to date no published information is available regarding this subject.

With regard to the transmission of tularemia in nature by Amblyomma americanum, the picture is not clear. In recent years a number of epidemiological findings have been accumulated which seem definitely to incriminate this tick as a vector. Byfield, et al (1945) observed 15 cases of tularemia within a period of 4 weeks in an army maneuver area located in Tennessee during the summer of 1943. These authors stated that nearly all cases had a history of exposure to ticks and in a survey of the area made by Norman Topping the only tick collected was Amblyomma americanum. The ticks

collected during this survey were sent to R. R. Parker and E. Francis, neither of whom was able to isolate Bacterium tularense. Bost, et al (1948) reviewed 61 cases of tularemia in the Ozark region and reported that 63 per cent of the cases were of tick borne origin, but they failed to indicate which species they thought to be the vector. Both Amblyomma americanum and Dermacentor variabilis occur in this area. However from personal observations made in this area during that time, the former species was much more abundant than the latter and was p robably the principal vector. Philip (unpublished data) had observed the same condition during the summer of 1938 while making a survey in this same area. Washburn, et al (1949) in a study of 704 cases of tularemia in Arkansas found 56 per cent of the cases were tick-borne and that 31 per cent were due to handling infected wild game.

From the review presented above it would appear that the "lone star tick" definitely enters into the picture of tularemia transmission in its native area, yet it seems remarkable that this tick has not been found infected in nature. Since published experimental data is lacking concerning this tick and tularemia transmission, it seemed logical to use it as a vector for this study, particularly since the writer feels that within the near future the role that this tick plays in nature will be made clear.

MATERIALS AND METHODS

The original supply of the "lone star tick" used in the following experiments was obtained as fully engorged females collected from various demestic animals in the Hatfield environs, Polk county, Arkansas, during field investigations in the spring and summer of 1948.

Amblyomma americanum has proved to be a tick which can be handled with ease in the laboratory, and all stages attached to their hosts readily throughout the year. All the stages were handled in the same manner and except for isolation, infected ticks were treated the same as the non-infected ones. Plate III illustrates the storage tube used for this tick, a device which is relatively simple and was found to be effective in handling the various stages. These tubes were of pyrex glass and measured 150 x 25 mm. As observed in the illustration the tube contained three cotton plugs and a cork. The two inner plugs were placed in the tube dry, and aside from making a chamber to confine the ticks, these two plugs helped maintain a more even humidity within the tube. The outer cotton plug at the free end of the tube was moistened at regular weekly intervals with water. This appeared to give the ticks adequate moisture and further helped to control any of the immature ticks which occasionally escaped beyond the inner plug.

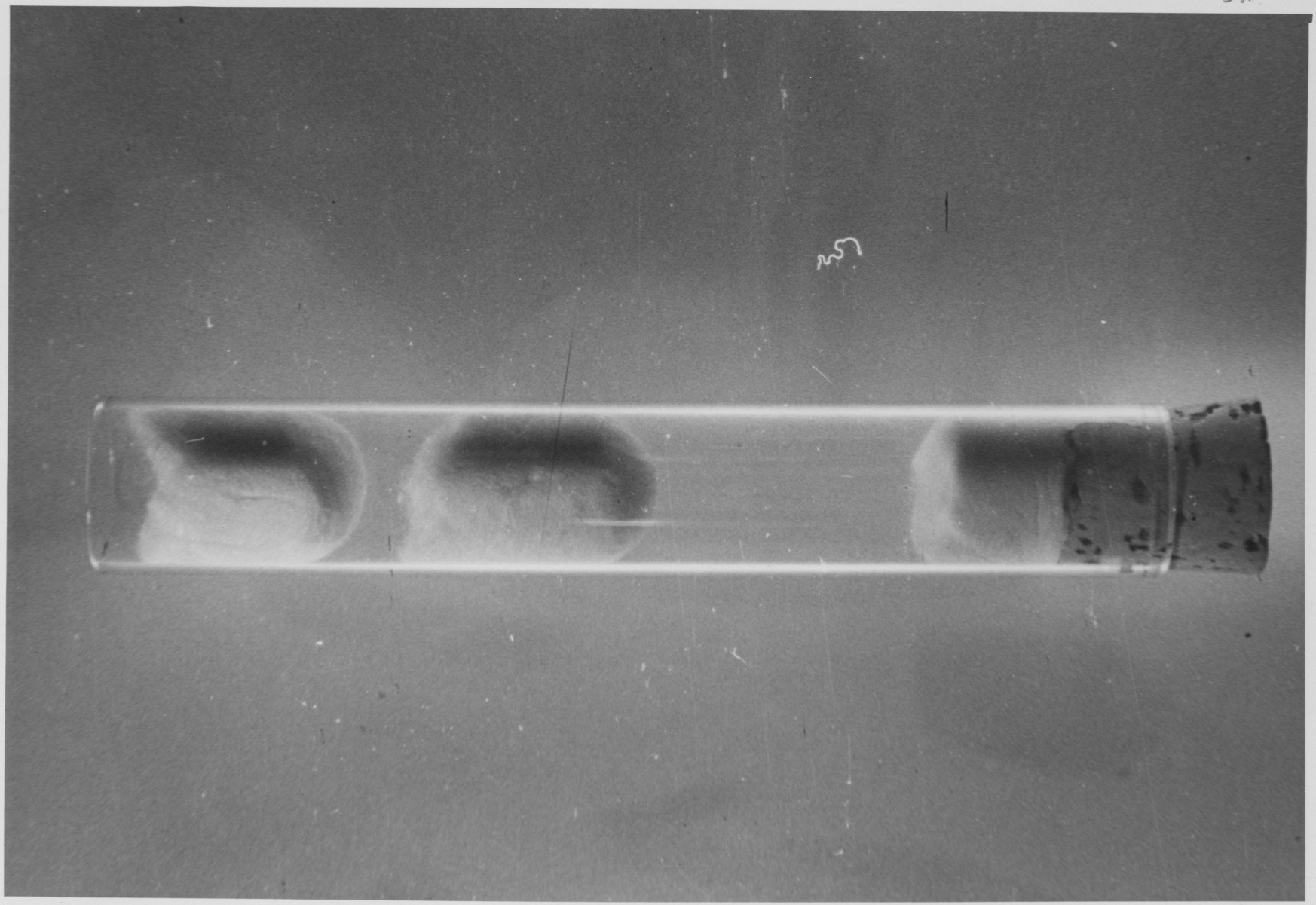

Plate III. Tick storage tube.

The cork was added to help maintain a more even humidity within the tube by preventing excessive evaporation, and it aided greatly in handling the various stages when in an unfed state. For example, if it was desired to concentrate the ticks at this end, the tube could be tapped on a solid surface without fear of breaking the tube and thus allow the ticks to escape. When the ticks were treated in this manner they were left at room temperature and humidity. However, these same tubes could be placed in humidifying jars containing a saturated solution of ammonium chloride which according to Peterson (1944) gives a relative humidity of 80 per cent. When this procedure was followed the corks were removed since observations indicated that too much moisture condensed inside of the tube and eventually caused considerable mortality among the ticks. Observations also indicated that Amblyomma americanum would do better when placed in the humidifying jars if the original wet plug was kept only slightly moist. If dependent upon the inorganic solution alone for humidity a higher per cent of mortality was observed, particularly during the moulting process. The ticks could go 3 to 4 weeks without further observation in the jars when the plug was moistened slightly.

Ticks to be stored over long periods of time were usually left at room temperature, since general observations indicated that this tick did not keep well when stored at 5 to 7° C. as reported

by Kohls (1935) for Dermacentor andersoni. From the time engorgement was complete until the moult to the subsequent stage was reached the ticks were kept at a temperature of 85° F. This procedure cut down the mortality between the various stages to 2 to 3 per cent in most cases and also shortened the periods between moults to 10 to 12 days.

The guinea pig was used as the animal of choice in feeding the immature stages. The animals used were of pure genetic strains obtained from the Endocrine Research Laboratory, University of Kansas. When it was desired to infect ticks either in the larval or nymphal stage the guinea pigs were injected with 1.0 ml of a 10^{-7} dilution of a standard suspension of a virulent culture (Sm) of Bacterium tularense 18 hours after infesting the animal with ticks. When doing experiments where the quantitative plate count method was used, guinea pigs of approximately 500 grams were used in an attempt to standardize procedures as much as possible.

Plate IV illustrates the method by which the ticks were fed during the larval and nymphal stages. The guinea pigs were placed in the holder which in turn was placed in a finely woven cloth sack. The ticks were then emptied from the storage tubes onto the guinea pig and the sack tied to prevent the ticks from escaping. After being in the sack from 12 to 24 hours the guinea pig and holder were removed and placed in an enamel pan which was filled to a

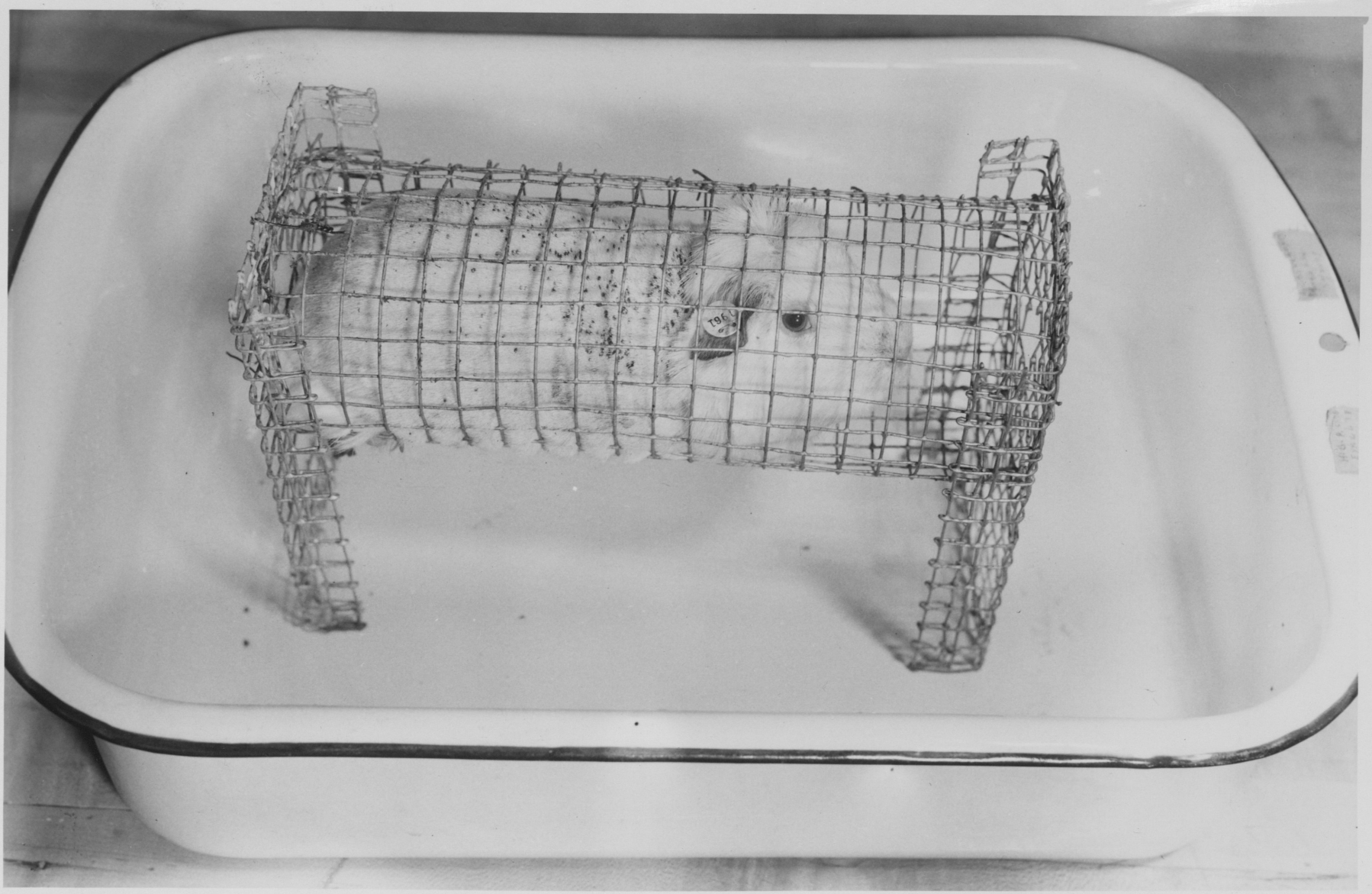

Plate IV. Method used in feeding the immature stages of Amblyomma americanum.

depth of 3 inches with water. This caused those few ticks which were not attached to become trapped in the water moat. As an additional precaution to keep ticks from escaping a double ring of vaseline was placed towards the upper edge of the pan. The sack which had contained the guinea pig and the ticks was then autoclaved at 30 lbs. pressure for 20 minutes to destroy any ticks remaining in the sack. During the 4 to 5 day period required for the larvae and nymphs to engorge, the guinea pig was fed a diet of cabbage and carrots to cut down the amount of fecal material in the water moat.

On completion of engorgement the ticks would fall into the water moat of the pan. By changing the water in the pan as the first ticks dropped and by clipping most of the hair from the guinea pig before placing it in the holder very little waste material was present at the time the ticks detached. The ticks were removed from the water every twelve hours by means of a large bulb syringe with an opening sufficiently large to draw up the engorged nymphs readily. The ticks were then placed in a large glass funnel lined with paper toweling. The funnel was then filled with water and agitated. This caused the ticks and the debris present to circulate in the water, and the ticks being heavier would settle to the bottom. As the ticks settled, the water and debris was drawn off by the bulb syringe used previously. By washing the ticks in this fashion 3 to 4 times very little debris was left and the ticks

were allowed to drain until dry as possible in the funnel. Upon completion of draining, the paper toweling and ticks were removed with the toweling being spread flat on a table top. The ticks were then transferred to storage tubes, the number per tube depending upon the experimental purpose for which they were intended.

The method of making quantitative plate counts was essentially that reported for the mites. However, the ticks were ground in 2 ml of saline instead of 1 ml. More difficulty was encountered in infecting the ticks since they did not engorge uniformly in regard to time and the guinea pigs died in an irregular manner. Therefore, in selecting ticks for this work in an effort to make procedures uniform, the death of the guinea pig as compared to the time most of the ticks engorged was taken into consideration. As an additional check a series of pools consisting of 5 ticks per pool were ground and injected into white mice in an effort to establish a rough index of the per cent infected. If such pools were negative, the supply of ticks from which they were selected was discarded.

RESULTS

Preliminary experiments were undertaken to verify the work of Philip which was cited by Parker (1933). These experiments readily revealed the ability of the "lone star tick" to be infected as

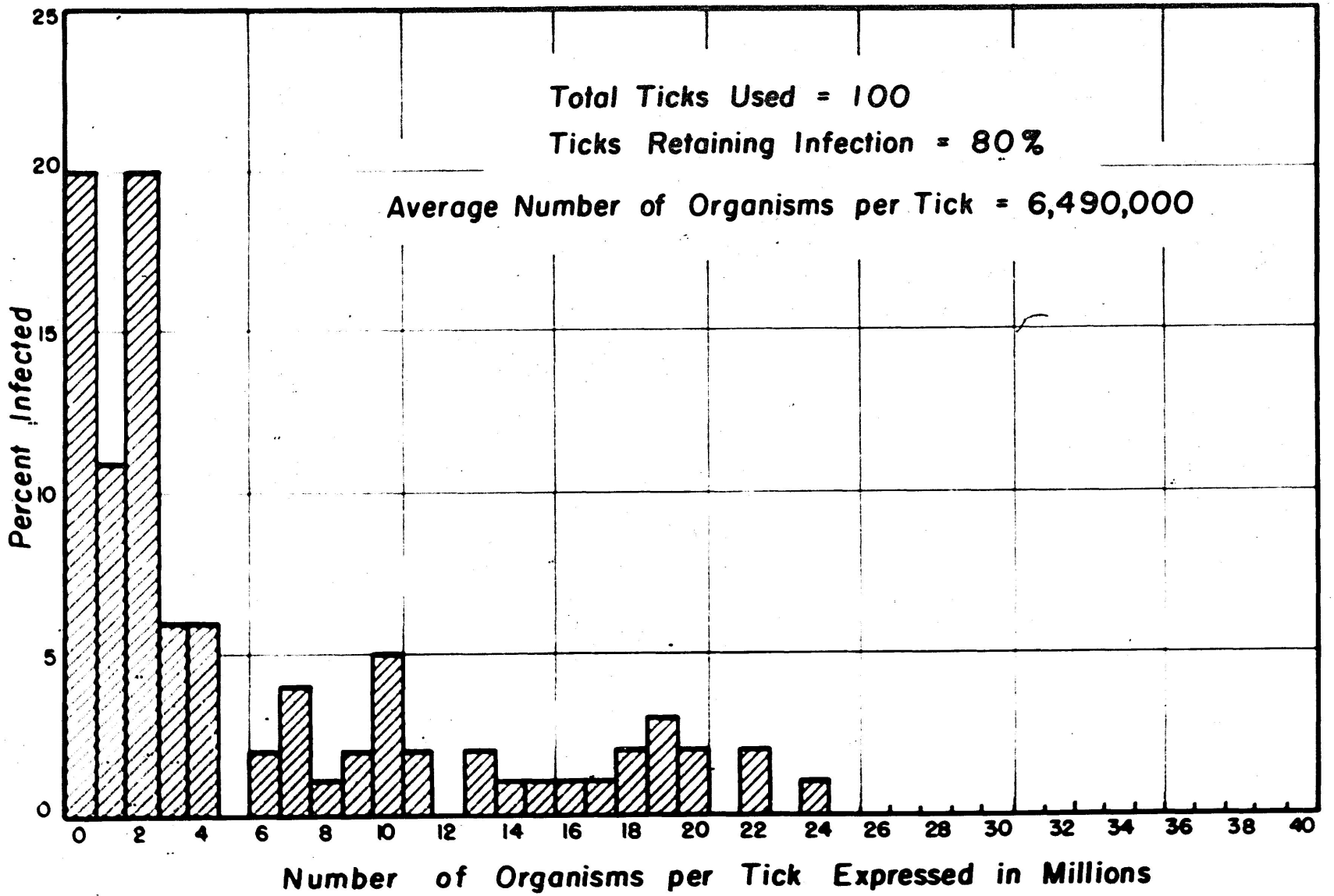

Fig. 4. Variation of the number of organisms per tick after one infective feeding.

larvae and to retain the infection through the nymphal stage and thence to the adult. No increase in mortality of the ticks was observed when compared to similar series of uninfected ticks fed and handled under the same conditions.

With the above information definitely established, a series of nymphal ticks was infected by feeding them on guinea pigs in an effort to establish the number of organisms per tick after moulting to the adult stage. These ticks were retained approximately 4 weeks after moulting before being ground for the quantitative plate counts. At this time 100 adults were removed from the various storage tubes. An equal number of females and males were used in this experiment to ascertain if the number of organisms would vary according to the sex.

Of the 100 ticks used 80 became infected and all infected ticks gave positive plate counts. Figure 4 shows the variation in the relative number of organisms per tick. The minimum and maximum numbers of organisms were 180 and 24,000,000 respectively while the average number was 6,490,000 per tick. These results indicated that the variation within a series of tics would be large, in fact larger than that reported in the case of the mites.

There is little information available concerning the ability of hard ticks to retain Bacterium tularense after prolonged periods of starvation. Parker (1924) reported from circumstantial evidence

that Dermacentor andersoni in one instance must have been able to retain the organisms for at least 8 months since the infected unfed adults were recovered in May of 1923 and he assumed they could not have fed later than the summer of 1922. Olsufiev (1943) reports that in starving mature ticks of the species Dermacentor marginatus Bacterium tularense retains its vital capacity and virulence about 1 1/2 years (530 days). No indication is given as to the temperature used to maintain the ticks nor does he mention the number of ticks used.

In view of these findings an experiment was undertaken to determine the number of organisms which could be recovered from infected adult Amblyomma americanum which had fasted for a period of 6 months at room temperature. For this purpose a small number of ticks were available from the same series utilized above to determine the number of organisms per tick.

Table III shows the results of the experiment, of the 10 ticks used 8 were positive by plate counts and 2 by mouse inoculation only. The minimum and maximum number of organisms per tick was 40 and 1,000,000 respectively while the average number was 176,040 per tick. This is a distinct decrease in the average number of organisms per tick as compared to those of the same lot which had been plated approximately 5 1/2 months previously. It is realized that this is too small a number of individuals to obtain conclusive

evidence. However, it does seem to be sufficient to suggest that upon prolonged starvation the ticks do not lose their infection, but the number of organisms per tick is reduced.

TABLE III

Survival of Bacterium tularense in fasting ticks

Number of Ticks	Number of Organisms Per Ticks	Death of Mouse In Days	Autopsy Plates
1	2,060	4	
2	36,000	3	
3	40	5	
4	98,000	3	
5		4	P*
6	1,000,000	3	
7	416,000	3	
8	480	4	
9	208,000	5	
10		5	P

* P indicates positive cultures at autopsy.

Observations were made on another group of ticks of this series to determine if starved ticks were capable of producing an infection

in susceptible animals. In this experiment 6 females and the same number of males were placed upon each of 15 guinea pigs. The guinea pigs died in from 4 to 10 days with 6.5 days as the average length of time. All autopsy plates were positive for Bacterium tularense.

The results indicated that ticks which had fasted for 6 months time produced infection in the animals as readily as ticks which had fasted for a period of only two weeks after reaching the adult stage. The comparison with the ticks which had fasted for the two week period were not run simultaneously, but are the results of a number of observations accumulated during the course of this study.

The effects of tick passage upon the virulence of Bacterium tularense has not been studied prior to this investigation. In an attempt to obtain evidence concerning this question mouse LD_{50} titration were made on organisms recovered from 4 adult ticks by the plate count method. One transfer was made from these original cultures before the titration was undertaken. The technique used was identical to that reported in the mite experiment.

The titration of the organisms from the 4 ticks were very uniform in nature and were as follows: $10^{-8.59}$, $10^{-8.74}$, $10^{-8.74}$, and $10^{-8.50}$. When compared to the titrations of the mite and the standard titration on this strain, the tick-passaged organisms are consistently lower. Whether this difference is large enough to

conclude that the tick passaged organisms were decreased in virulence is a questionable matter. More investigation of this factor is required before definite conclusion can be drawn; however, these particular results suggest that a decrease in the virulence is possible.

Bell (1945) in studying the retention of tularemia by Dermacentor variabilis concluded that "ticks feeding on immune or normal hosts lost their infection, presumably as a result of the stimulating effect of the blood meal upon a normal bactericidal function of the ticks gut, but before losing their infection as a result of feeding, infected ticks may inoculate the host, where upon if it is not immune will develop septicemia and infect all ticks feeding upon it while infected ticks feeding on immune animals permanently lost their infection."

This author used vaccinated domestic rabbits, for the most part, as his supply of "immune" animals. This is surprising since Downs, et al (1932) found that domestic rabbits vaccinated with formalin killed cultures of Bacterium tularense developed only a slight resistance to subsequent infections and that they were not immune in the sense that they did not resist challenge with even a small number of living virulent organisms although they had an abundance of circulating antibodies. Inasmuch as Bell stated that two rabbits had received a total of 12 ml of vaccine without

specifying the type and failed to report an antibody titer for the rabbits used it seemed probable that worthwhile information could be gained by repeating this experiment under more controlled conditions.

In an effort to collect this information a series of larvae were infected by feeding upon a guinea pig which had been innoculated by the method described above. On moulting to the nymphal stage 100 ticks were ground individually and injected into white mice and 83 per cent were found to be infected. The remaining nymphs were placed upon domestic rabbits which had an antibody titer of 1 : 2560 as demonstrated by the agglutination test. This antibody titer was produced by injecting the animal with 2 ml of a formalized suspension of the Sm strain intraperitoneally every other day until a total of 3 innoculations had been received. Upon completion of the injections a period of 10 days elapsed before a blood sample was withdrawn for the demonstration of agglutinins. The rabbit was placed in a holder similar to that described for the guinea pig. The rabbit was allowed a normal diet until the ticks became engorged at which time it was fed cabbage and carrots for the last 2 days it remained confined. By the sixth day all nymphs had engorged and dropped from the host. On moulting to the adult stage the ticks were retained a period of 10 days at which time 100 were removed from the storage tubes and quantitative plate counts were made of each tick.

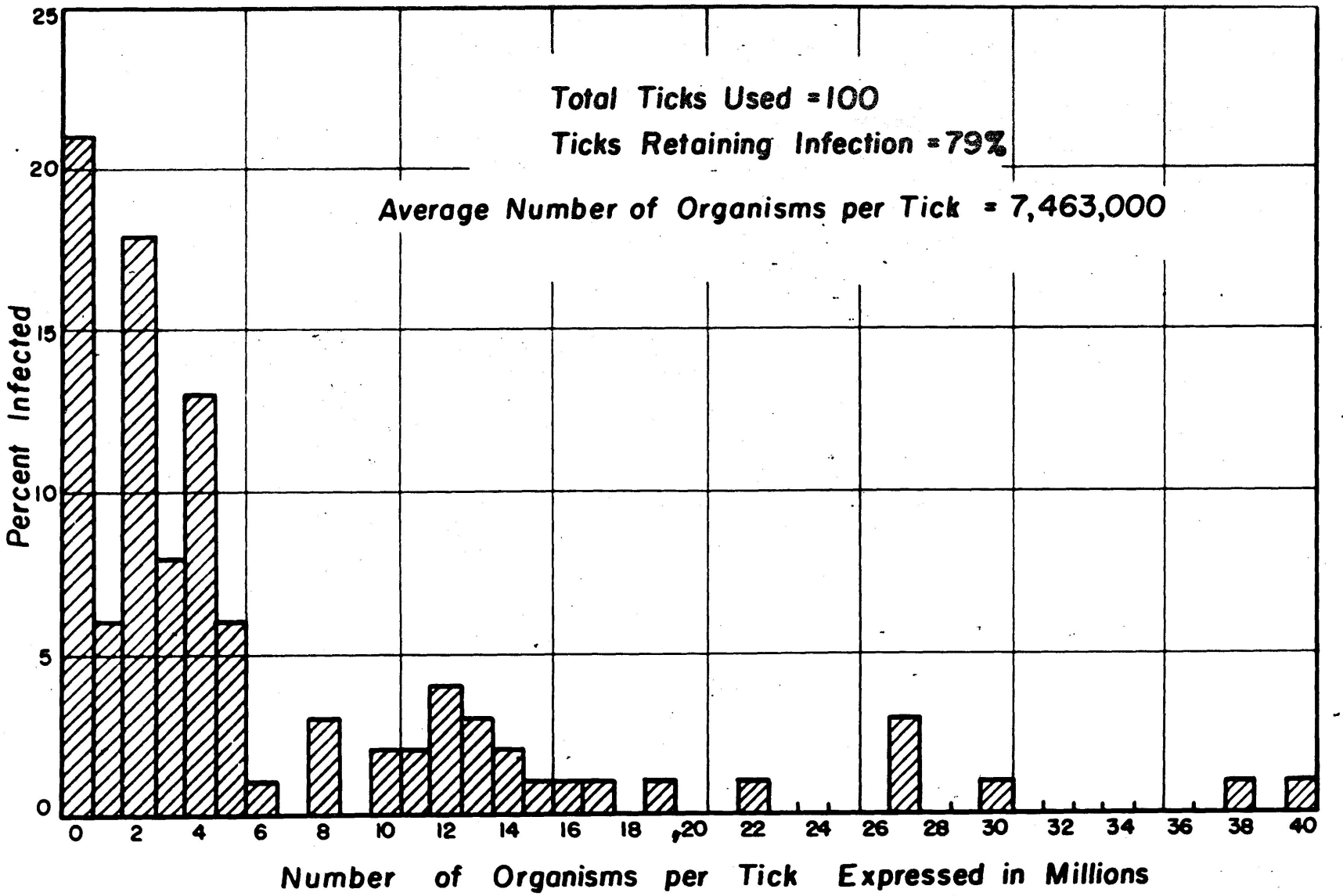

Fig. 5. Variation of the number of organisms from infected ticks after feeding on a vaccinated rabbit.

Seventy-nine per cent of the ticks proved to be infected. Figure 5 shows the variation in the number of organisms per tick. The minimum and maximum number of organisms was 600,000 and 40,000,000 respectively while the average number was 7,463,000. The reduction of 6 per cent in the number of infected adult ticks as compared to the unfed nymphs is not believed to be significant. The results of this experiment are a complete reversal to those reported by Bell since the number of infected ticks in this experiment was not decreased. The average number of organisms per tick compares favorably with the earlier experiment where the number of organisms were calculated for the first time. However, it is not known whether the ticks which fed on the vaccinated rabbit experienced an increase or decrease in the number of organisms due to the feeding since the number of organisms per tick was not determined before ticks were placed on the vaccinated rabbit.

In view of the above results a series of experiments were undertaken to determine the effects of feeding ticks infected with Bacterium tularense upon naturally resistant and recovered hosts. Such a study is particularly important because under natural conditions infected ticks of this species must feed regularly on resistant or immune animals. If there were a change in the number of organisms, or if the number of infected ticks decreased, this

information would be of considerable importance regarding this type of disease transmission in nature. On the other hand, if no apparent change in the number of organisms could be observed, this fact also is of importance.

For this purpose a large number of larvae were infected and after moulting to the nymphal stage the ticks were allowed to fast for one week. To determine the number of organisms per tick and the percentage of ticks infected before they were fed on resistant or recovered animals, 300 unfed nymphs were removed from the various storage tubes and the following procedures were carried out. The ticks were divided into 3 series of 100 ticks each. Series A_1 was further divided into 5 units with each unite consisting of 20 ticks; series B_1 was divided into 10 units with each unit consisting of 10 ticks and the remaining ticks (series C_1) were considered as individual units. Each unit from the various series was ground in 2 ml of saline with mouse inoculations and plate counts made in the usual manner. The two additional series (A and B) were used in an attempt to obtain more information on the average number of organisms per tick. Since the infected ticks could not be separated from the non-infected the use of these pools had no value in determining the percent infected. This same procedure was also used on the ticks recovered from the various hosts in the later experiments except in making the plate counts,

the greater concentration of ticks in the units of series A and B made it necessary to make the dilutions to 10^{-6} before satisfactory counts could be obtained.

Table IV illustrates the results of series A_1 and B_1. The average number of organisms per tick for these two series are relatively the same as is the minimum and maximum range. In series C_1 74 per cent of the ticks used proved to be infected. Figure 6 shows the variation in the relative number of organisms per tick, and the minimum and maximum number was 4,000 and 100,000,000 while the average for the infected ticks was 4,226,000. In view of these findings it seemed that the average number of organisms for the three series was within the normal range of variation and that the number of organisms per tick was well established before being placed on the various hosts.

After the per cent infected and number of organisms had been determined for the unfed nymphs, a sufficient number of them were placed on naturally resistant and various recovered animals to insure that at least 300 ticks would be obtained from each type of host after moulting to the adult stage. The ticks were placed on the various hosts at the same time and in all subsequent procedures were handled essentially as in the unfed nymphs reported above, the comparable series of ticks being identified as A, B, and C, with differentiating numerals.

Quantitative plate counts on unfed nymphs before being placed on the various hosts - (Series A and B).

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	20	15,600,000	780,000
2	20	26,600,000	1,330,000
3	20	100,000,000	5,000,000
4	20	200,000,000	10,000,000
5	20	100,000,000	5,000,000
Total	100	442,200,000	4,422,000

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	10	3,600,000	360,000
2	10	7,800,000	780,000
3	10	13,400,000	1,340,000
4	10	22,400,000	2,240,000
5	10	44,800,000	4,480,000
6	10	32,000,000	3,200,000
7	10	50,000,000	5,000,000
8	10	36,000,000	3,600,000
9	10	100,000,000	10,000,000
10	10	60,000,000	6,000,000
Total	100	370,000,000	3,700,000

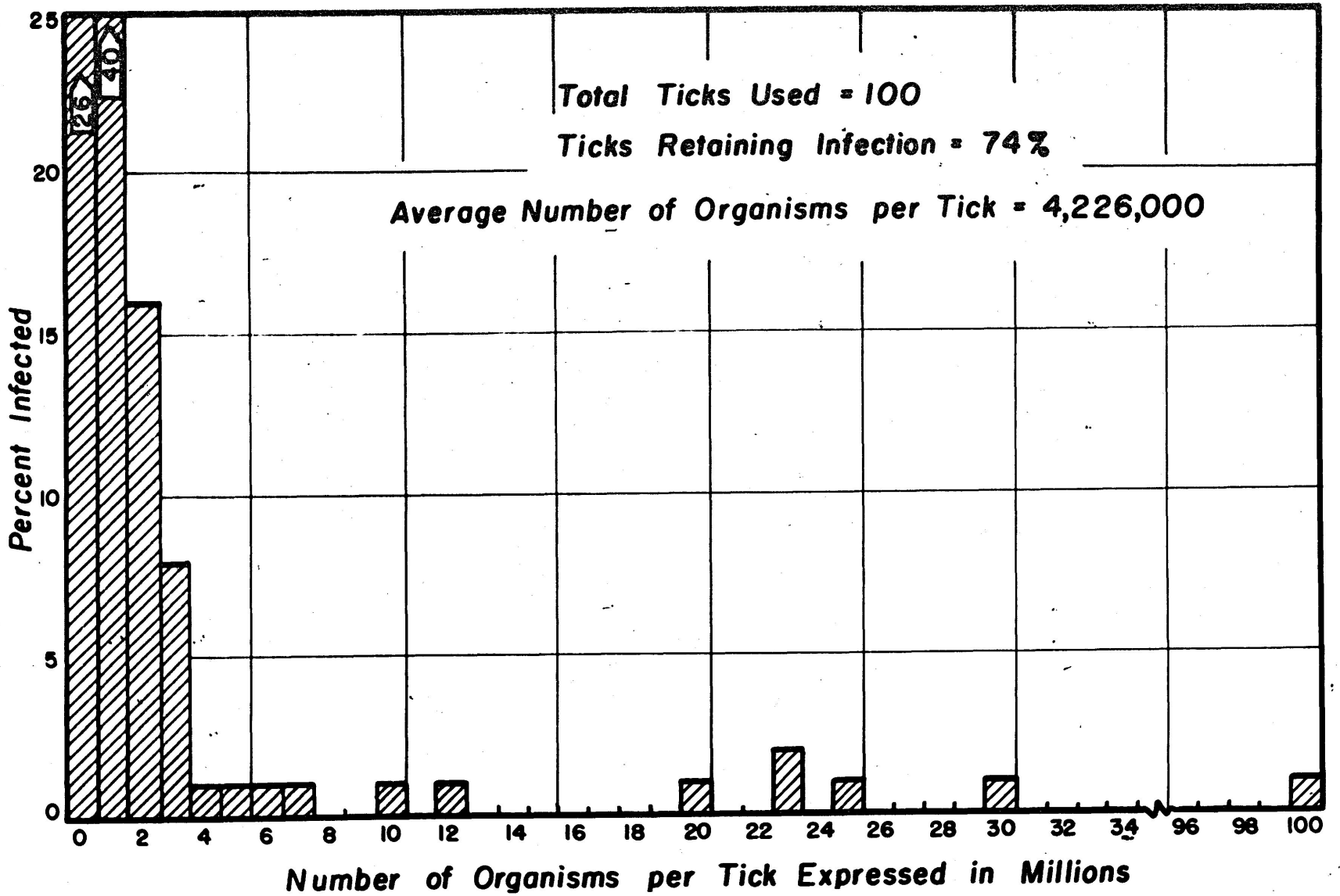

Fig. 6. Variation of the number of organisms from infected unfed nymphs.

Dogs were the animal of choice in studying the effects of feeding infected ticks upon naturally resistant hosts since they are usually considered resistant to tularemia. However, Ey and Daniels (1941) reported the spontaneous occurrence of tularemia in dogs, the infection of which apparently was produced as a result of eating infected wild rabbits. Johnson (1944) isolated Bacterium tularense from a pool of spleens from dogs ill with canine distemper. He inoculated 42 dogs by various routes and although they all became infected only 9 died. Downs, et al (1947_a) in testing the susceptibility of various animals found dogs to be much less susceptible than any of the other animals used and fatalities were produced only after overwhelming doses were given.

For this experiment a normal female dog pup approximately three months old was used. This animal had been reared in the laboratory for two months and a blood sample withdrawn shortly before the ticks attached was negative for the agglutinins of Bacterium tularense. At the end of the fourth day after attachment, approximately 800 fully engorged nymphs were recovered from this animal.

Table V illustrates the results of series A_2 and B_2. The average number of organisms of both series was greater than had been expected. With the exception of the fourth pool in series A_2 the average number of organisms per tick in both series was relatively

Quantitative plate counts on ticks after feeding on naturally resistant host - (Series A and B).

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	20	2,420,000,000	121,000,000
2	20	400,000,000	20,000,000
3	20	680,000,000	34,000,000
4	20	40,000,000	2,000,000
5	20	1,280,000,000	64,000,000
Total	100	4,820,000,000	48,200,000

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	10	200,000,000	20,000,000
2	10	400,000,000	40,000,000
3	10	400,000,000	40,000,000
4	10	300,000,000	30,000,000
5	10	400,000,000	40,000,000
6	10	500,000,000	50,000,000
7	10	200,000,000	20,000,000
8	10	400,000,000	40,000,000
9	10	280,000,000	28,000,000
10	10	400,000,000	40,000,000
Total	100	3,480,000,000	34,800,000

uniform. Seventy per cent of the ticks in series C_2 proved to be infected. Figure 7 gives the variation in the relative number of organisms per tick. The minimum and maximum number of organisms was 12,000 and 200,000,000 while the average number of organisms for the infected ticks was 29,600,000. A blood sample was withdrawn from the dog three weeks after the ticks had detached. At this time the antibody titer was 1 : 320. At no time while the ticks were attached or afterwards did this animal show any symptoms.

In feeding infected ticks upon recovered animals, two different kinds of hosts were used; namely: a domestic rabbit which had recovered from a laboratory infection of a naturally low virulent strain of Bacterium tularense and white rats which had been actively immunized by receiving a sub-lethal dose of fully virulent organisms.

According to Meyer (1949) rabbits which occasionally recover from experimental infections as a rule are resistant to many fatal doses. The rabbit utilized in this experiment had been infected with 1 ml of 10^{-9} dilution of a standard suspension injected intraperitoneally. The organisms used were of a strain of Bacterium tularense which in mouse and guinea pig LD_{50} titrations appeared to be as fully virulent as the Sm strain. A blood sample was withdrawn from this rabbit one week before the ticks were allowed to attach and an antibody titer of 1 : 2560 was demonstrated by means of the agglutination test. Inasmuch as rabbits do not survive

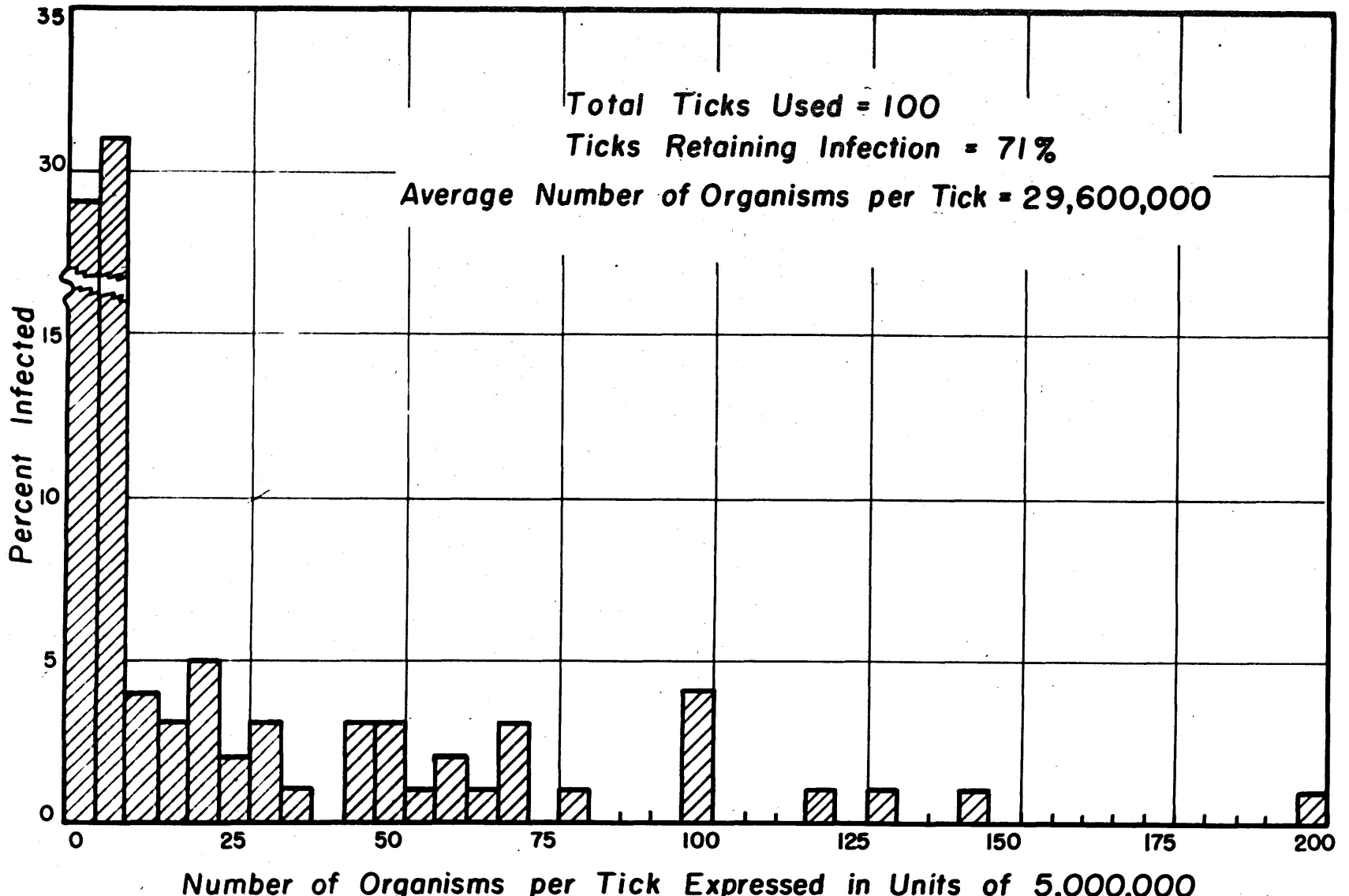

Fig. 7. Variation of the number of organisms in infected ticks after feeding on a naturally resistant host.

injection of 1.0 ml of the Sm strain intraperitoneally it was assumed that this rabbit had recovered from infection with a strain of lower virulence for rabbits. By the end of the fifth day approximately 400 fully engorged nymphs were recovered from the rabbit and about 12 hours later the rabbit died. Autopsy was performed and plate cultures made of the spleen and heart blood. The gross findings in the rabbit during autopsy were negative for tularemia with the spleen, liver, and lymph nodes appearing normal. The culture plates however were positive and mice injected with organisms from these cultures were dead within 48 hours. Autopsy cultures of these mice proved to be positive for Bacterium tularense and it was assumed that the rabbit died acutely of tularemia.

Table VI shows the results of feeding series A_3 and B_3 on the recovered rabbit. The average number of organisms per tick for both series differed by 15,000,000 organisms per tick. However with this large number of organisms per tick it seems probable that a good deal of variation would be expected. Eighty-six per cent of series C_3 proved to be infected and figure 8 illustrates the variation in the number of organisms per tick. The minimum and maximum was 200,000 and 200,000,000 respectively while the average number of organisms for the infected ticks was 41,600,000.

White rats are known to survive following the injection of a

TABLE VI

Quantitative plate counts on ticks after feeding on a rabbit recovered from a low virulent strain of *Bacterium tularense*. (Series A and B).

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	20	460,000,000	23,000,000
2	20	1,640,000,000	82,000,000
3	20	1,260,000,000	63,000,000
4	20	280,000,000	14,000,000
5	20	2,440,000,000	122,000,000
Total	100	6,080,000,000	60,800,000

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	10	780,000,000	78,000,000
2	10	1,260,000,000	126,000,000
3	10	340,000,000	34,000,000
4	10	660,000,000	66,000,000
5	10	640,000,000	64,000,000
6	10	320,000,000	32,000,000
7	10	140,000,000	14,000,000
8	10	240,000,000	24,000,000
9	10	1,360,000,000	136,000,000
10	10	1,760,000,000	176,000,000
Total	100	7,500,000,000	75,000,000

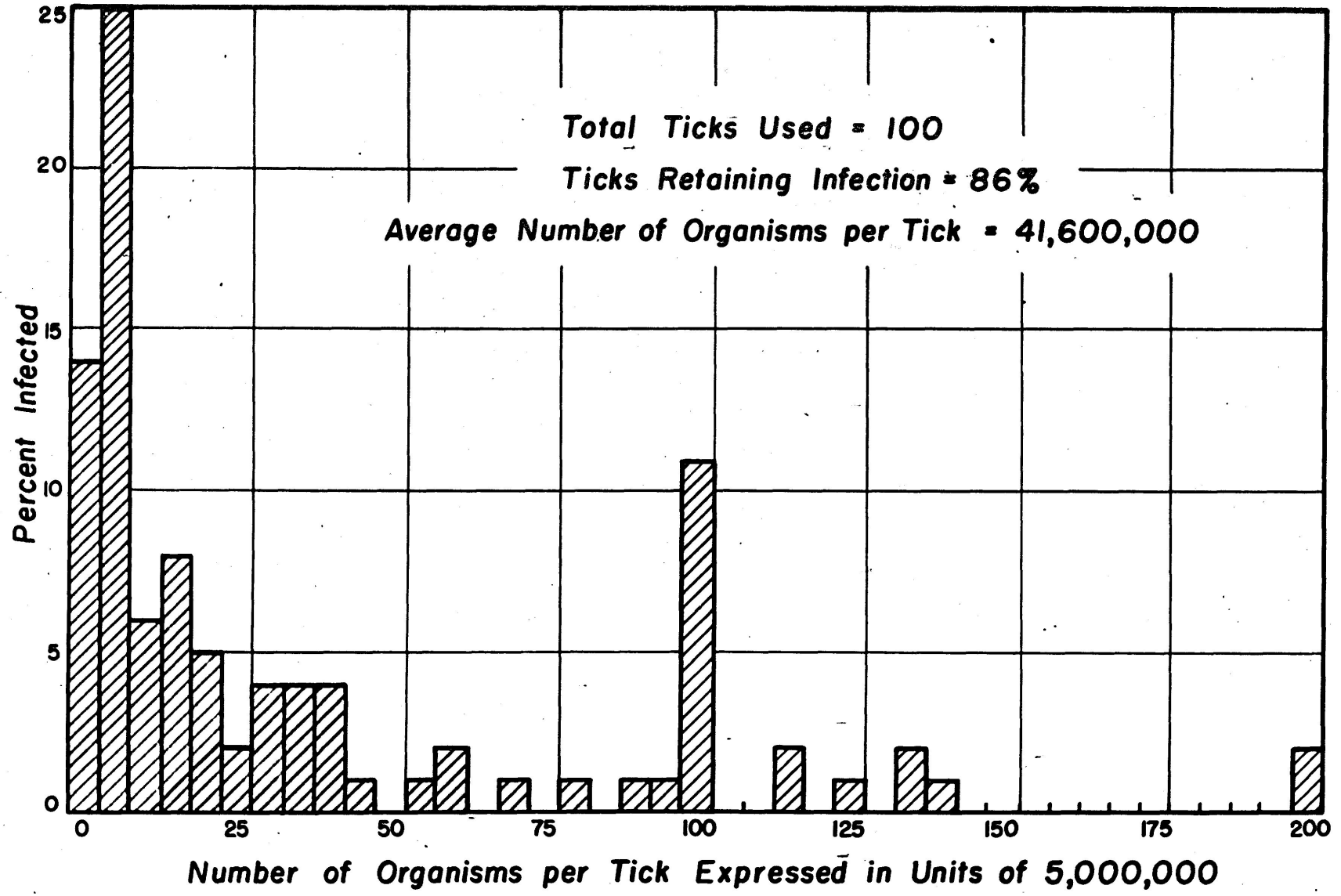

Fig. 8. Variation of the number of organisms from infected ticks after feeding on a recovered rabbit.

small number of virulent organisms, (Downs, et al 1947a) and upon recovery they resist challenge with 10,000 to one million LD_{50} doses, (Downs, et al 1947b). Subsequent work by Downs, Buchele, and Edgar (1949), and Buchele and Downs (1949) have verified the earlier work and they have shown that the animals which recovered from an infection were more immune than those rats protected by vaccines.

The rats used in this experiment were assumed to be solidly immune since these animals were infected by injection of 1 ml of a 10^{-6} dilution of a standard suspension of the Sm strain. Upon recovery these animals resisted challenge with a 1000 LD_{50}. After this challenge a number of the rats were bled to death, and the blood was pooled for agglutination tests. An antibody titer of 1 : 640 was obtained for Bacterium tularense. One week later a booster shot of 1 ml of a 10^{-2} dilution of a standard suspension of the Sm strain was injected intraperitoneally. Nine days after receiving this booster shot the unfed nymphs were allowed to attach upon a series of 20 rats.

The attachment of the ticks to the rats was not accomplished with the success encountered when using dogs, rabbits, or guinea pigs. The number of ticks which attached to the various rats varied from 18 to 105. An average of 7 to 8 days was required for engorgement on this animal as compared to 4 to 5 days for those ticks feeding on rabbits or guinea pigs. The same extra period of

time was required for the moulting process to take place even though conditions of temperature and humidity were identical. Approximately 800 engorged nymphs were recovered from the rats and it was observed that within one day many of the ticks appeared to be in a moribund state. At the end of a weeks time approximately 80 per cent of the ticks were in this condition which made it impossible to use normal active ticks for the plate counts.

Table VII contains the results of the plate cultures made of series A_4 and B_4 for the ticks recovered after feeding on the solidly immune rats. Only a slight difference was found in the average number of organisms between the two series. Sixty-three per cent of the ticks used in series C_4 proved to be infected and figure 9 illustrates the variation in the relative number of organisms per tick. The minimum and maximum number of organisms was 52,000,000 and 110,400,000 respectively while the average number for the infected ticks was 20,700,000.

As a control a group of the remaining unfed nymphs were fed upon non-infected guinea pigs. This group of nymphs consisted of ticks which failed to attach to the recovered rats in the above experiment.

Table VIII illustrates the results of the plate counts after the infected ticks had fed on normal susceptible hosts for series A_5 and B_5. The difference between the average number of organisms

TABLE VII

Quantitative plate counts on ticks after feeding on recovered hosts - (Series A and B).

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	20	240,000,000	12,000,000
2	20	1,340,000,000	67,000,000
3	20	1,100,000,000	55,000,000
4	20	640,000,000	32,000,000
5	20	860,000,000	43,000,000
Total	100	4,180,000,000	41,800,000

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	10	40,000,000	4,000,000
2	10	760,000,000	76,000,000
3	10	280,000,000	28,000,000
4	10	900,000,000	90,000,000
5	10	200,000,000	20,000,000
6	10	840,000,000	84,000,000
7	10	320,000,000	32,000,000
8	10	300,000,000	30,000,000
9	10	320,000,000	32,000,000
10	10	620,000,000	62,000,000
Total	100	4,580,000,000	45,800,000

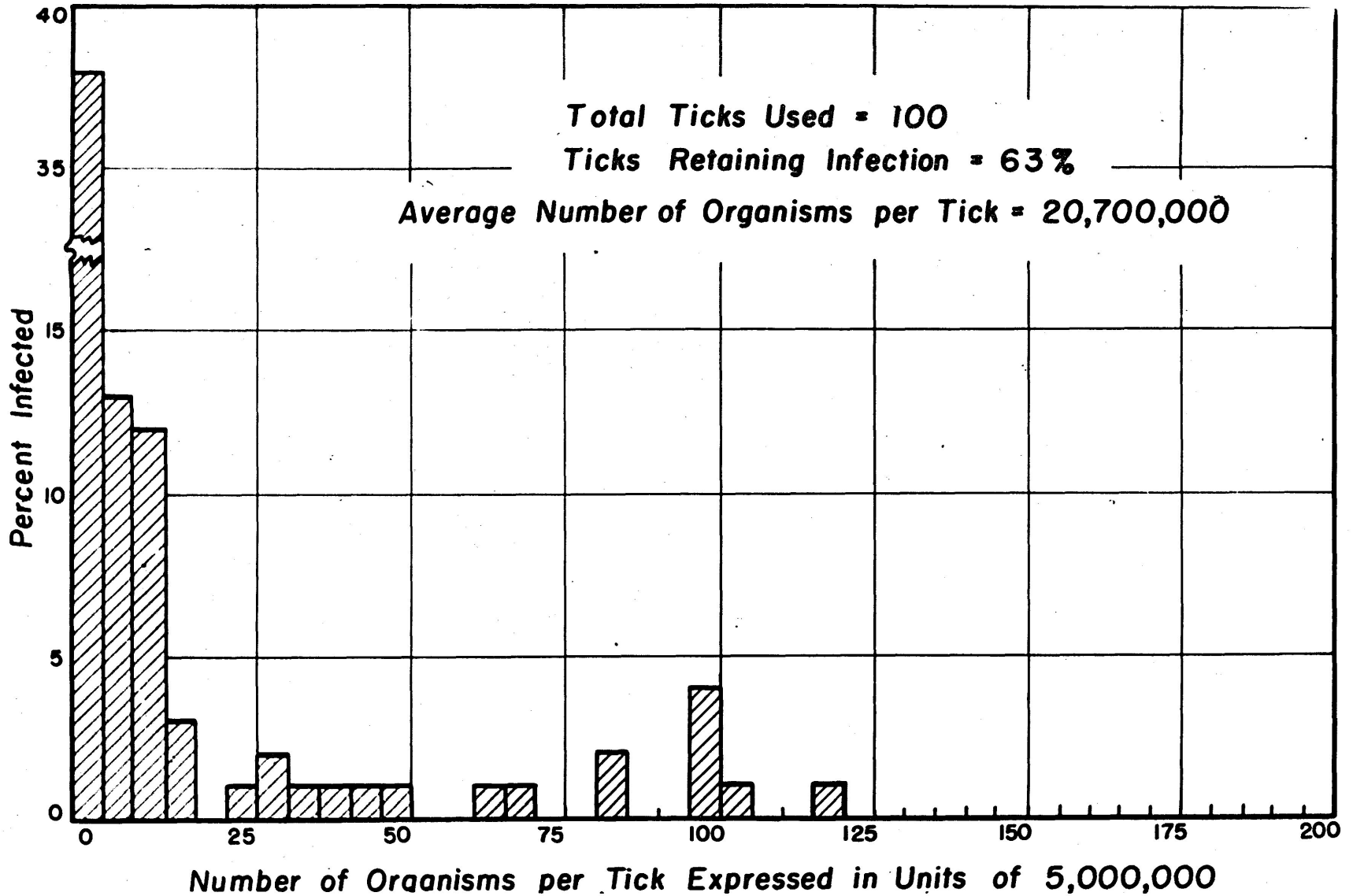

Fig. 9. Variation of the number of organisms from infected ticks after feeding on recovered rats.

per tick for the two respective pools are not of particular importance, where such a large number of organisms are concerned. Sixty-eight per cent of the ticks used in series C_5 were found to be infected and figure 10 shows the variation in the relative number of organisms per tick. The minimum and maximum number was 44,000 and 204,800,000 respectively while the average number for the infected ticks was 78,100,000. As observed in figure 10, over 50 per cent of the ticks infected had 100,000,000 or more organisms, yet none of the ticks had a maximum number equal to the average of series A_5 or B_5. A similar condition was not observed in the ticks obtained from the other animals and due to the unexpected increase in the number of organisms the 10^{-4} dilution was not sufficient. If the ticks in this particular C_5 series had been carried out to a 10^{-6} dilution as used in all A and B series it is believed that the higher number of organisms would have been more varied and probably would have extended far beyond the maximum number reported.

The wide gap in the average number of organisms for the A and B series as compared to C after the ticks reached the adult stage was not expected and is difficult to explain since the cause for the difference is not known. However, the A and B pools show the same general trend as do the C series and for this reason are of value. The difference between the A and B series in comparison to C may possibly be explained in the following manner: since all

TABLE VIII

Quantitative plate counts on ticks after feeding on susceptible hosts - (Series A and B).

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	20	4,120,000,000	206,000,000
2	20	3,300,000,000	165,000,000
3	20	5,280,000,000	264,000,000
4	20	4,100,000,000	205,000,000
5	20	5,720,000,000	286,000,000
Total	100	22,520,000,000	225,200,000

Number of Pool	Number of Ticks	Number of Organisms Per Pool	Average No. Organisms Per Tick
1	10	380,000,000	38,000,000
2	10	1,720,000,000	172,000,000
3	10	2,580,000,000	258,000,000
4	10	2,000,000,000	200,000,000
5	10	580,000,000	58,000,000
6	10	2,380,000,000	238,000,000
7	10	1,700,000,000	170,000,000
8	10	2,700,000,000	270,000,000
9	10	2,940,000,000	294,000,000
10	10	3,600,000,000	360,000,000
Total	100	20,580,000,000	205,800,000

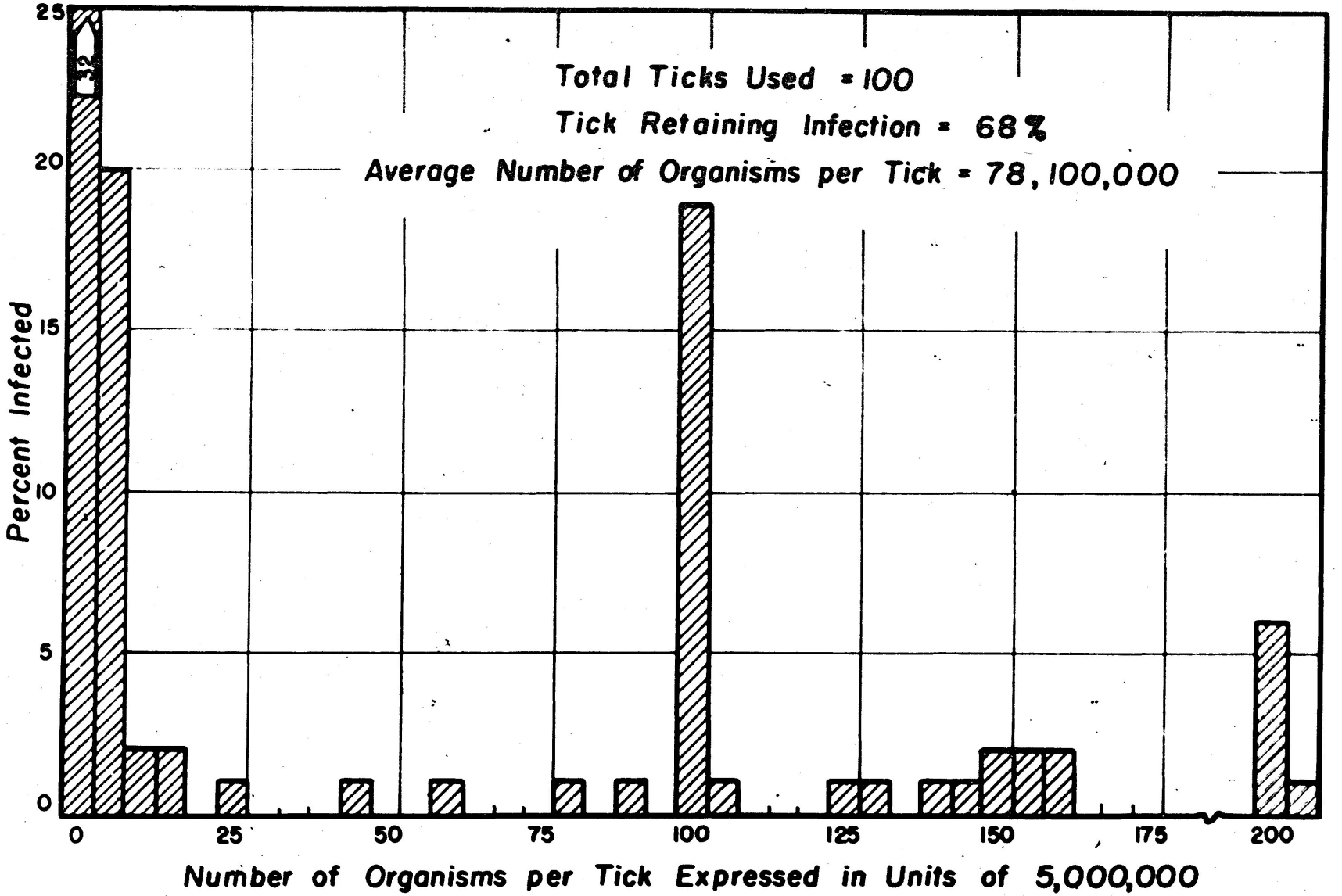

Fig. 10. Variation of the number of organisms from infected ticks after feeding on susceptible hosts.

units for the various series were ground in 2 ml of saline a great difference in the concentration of 10^{-0} suspension was brought about for series A and B as compared to C. In other words, since the 100 ticks were used in each series, A was ground in a total of 10 ml, B in 20 ml, and C in 200 ml of saline, with 5, 10, and 100 samples respectively taken from each series. It may be argued that since series A and B were more concentrated, the sample removed from the original suspensions for further dilutions would be more uniform and therefore present a clearer picture of the number of organisms per tick. On the other hand, admitting that the samplings from the C series would tend to be more variable, it seems probable that 100 samplings compared to 5 and 10 for the other two series would overcome the difference in concentration, and that any mechanical error in the process of sampling would not be exaggerated to the extent that it would in the other two series. Originally it was thought that the greater concentration of the pools would not matter. It was believed that by carrying them out to a higher dilution they would give approximately the same number of organisms per tick.

DISCUSSION OF TICK WORK

The experimental work reported above shows that a relatively high per cent of Amblyomma americanum can be infected, yet a good

deal of variation was observed. It seems probable that this is a condition which would be expected, particularly when three variables are involved; that of the host, the organism, and the vector. Yet despite these variabiles it is believed that the results presented have been clear cut and extend the knowledge of this type of disease transmission.

With regard to the evidence collected concerning the retention of organisms after prolonged periods of fasting it seems that the number of organisms were decreased but the per cent infected was not changed. That similar ticks could infect guinea pigs while feeding was clearly shown, and it indicates that in nature ticks which overwinter probably retain the infection, particularly if the results reported by Olsufiev (1943) can be varified in subsequent studies.

The LD_{50} titration studies indicated that the organisms passed through ticks were consistently lower in virulence than the mite or mouse Sm strains. As to whether this difference of one log is enough to support a conclusion that virulence is regularly decreased is doubtful. Further study must be undertaken concerning this factor before a definite statement is made.

The results of feeding infected ticks upon the vaccinated rabbits do not agree with the findings reported by Bell (1945). The reasons for the disagreement are not evident, particularly since the present study was done with a species of tick belonging

to a different genus than the vector used by Bell. Critical studies have not been made with the various ticks concerned in the transmission of tularemia and it is not improbable that specific and generic differences may occur here as was found in the extensive investigation of such diseases as yellow fever and malaria. However, since Bell generalized regarding all ticks the present study definitely refutes his claim that the ticks feeding on immune hosts permanently lose their infection. As to what this author meant by the term immune is not known since he did not give his opinion. Throughout the course of this study, the word immune has been avoided as much as possible. For the purpose of this study vaccinated rabbits were not considered immune since they do not resist challenge with even a small number of virulent organisms although they may have a high titer of circulating antibodies. If the word "immune" had been used in this study it would have been applied to the rats, since they can resist challenge with one million LD_{50} after recovery from a sublethal dose of virulent organisms. Since these ticks did not lose their infection, in fact showed a multiplication of the organisms, the present writer cannot agree with the former author, especially since it is believed that he did not use an "immune" animal in the sense that would resist challenge by fully virulent organisms. During the course of this study it was never observed that all ticks feeding on a susceptible

host became infected. This last statement was clearly demonstrated in the control group of infected nymphs, which after moulting to the adult stage were found to be 68 per cent infected. Compared to the ticks which fed on the recovered rats a difference of only 5 per cent was noted. This is especially interesting since the ticks which fed on the susceptible animals (guinea pigs) were specimens which failed to attach to the rats. In fact the average per cent of infection for the ticks which were recovered from the various hosts used in the experimental work concerned with the retention of the infectious organisms was 72 per cent as compared to 74 per cent before they were placed on the various hosts. From this it seemed that the nymphs had engorged and detached before the susceptible animals were circulating a sufficient number of organisms to influence the rate of infection. On the other hand, it may be possible that the ticks which were not infected as nymphs resist infection as adults; however, nothing is known concerning this particular point and it does not seem too probable.

It appears that the multiplication which took place in the ticks which fed on the various hosts was controlled somewhat by the degree of resistance each type of animal exhibited. In this particular case those ticks which fed on the solidly immune rats had the lowest average number of organisms, those which fed on the dog (naturally resistant) the next lowest, and the ticks from the

recovered rabbit and the susceptible hosts (guinea pigs) had the highest average number of organisms. The difference in the amount of multiplication in those ticks which fed on the rats and the dog was not great. Inasmuch as a large number of the ticks which fed on the rats reacted so unfavorably to this host, the difference between the two may be more apparent than real.

The results of feeding the ticks on the various hosts are of particular importance in the study of tularemia transmission by Amblyomma americanum in nature. For example, since the ticks did not lose their infection while feeding on extremely resistant animals, it seems probable that any of the hosts from which this species is recovered in the field would not have any influence upon the per cent of ticks infected. Therefore, ticks recovered from these animals could be used in the survey of the incidence of tularemia in nature.

GENERAL DISCUSSION

The results of the above studies have indicated that approximately the same percentage of mites and ticks became infected. This is especially interesting since the two vectors have widely separated feeding habits. The number of organisms in the adult ticks was greater than that in the mites. In ticks which were infected as larvae and plate cultures made of the unfed nymphs the difference in the number of organisms in this stage and the mites was not great.

When infected vectors are fed on various hosts, the mites showed relatively little increase in the number of organisms as compared to the ticks.

The Sm strain of Bacterium tularense was passaged through adult mites without change in virulence while the tick passaged material was consistently of lower virulence. It was interesting to observe that the one pool of mite protonymphs used in a LD_{50} titration was within the range observed in the titration of tick passaged material.

After prolonged periods of starvation both vectors were able to remain infected with no change in the number of individuals infected. In the case of the mites no change in the number of organisms was observed but a decrease was noted in the ticks.

SUMMARY AND CONCLUSIONS

1. Quantitative methods of study were used during the course of this study in an attempt to observe the changes which took place within certain infected arthropod vectors after prolonged periods of starvation and after feeding on various types of hosts.

2. The mite, Liponyssus bacoti, has been found capable of retaining an infection of Bacterium tularense from the protonymph stage throughout the subsequent stages of a single generation and in certain instances it transmits the infection to the next

generation. The infection was also retained after several feedings on recovered mice without a reduction of the number of organisms per mite or in the per cent infected.

3. Both Liponyssus bacoti and, the tick, Amblyomma americanum were able to remain infected after long periods of starvation. In the case of the mite no apparent loss in the number of organisms was observed, but the number of organisms was decreased in the tick.

4. The LD_{50} titration indicated no loss of virulence in the Sm strain of Bacterium tularense or mite passage. In the tick a lower LD_{50} was observed.

5. In feeding infected Amblyomma americanum upon various recovered and naturally resistant hosts, no change in the per cent infected was observed. A definite increase in the number of organisms per tick was demonstrated. This multiplication was not as great as that which occurred after infected ticks were fed on susceptible hosts.

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